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A NOTE ON THE BILIRUBIN CONTENT OF BLOOD AND UROBILINOGEN CONTENT OF URINE IN DIABETES MELLITUS.

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THERE is much to support the view that there is an intimate relationship between disease of the liver and diabetes mellitus. As a matter of fact, one of the oldest references to the pathology of diabetes was concerned with the liver; Mead (1748) first drew attention to the marked fatty infiltration. A vast amount of literature has accumulated on this subject since then; and, of all of the newer evidence, perhaps, the most striking is the observation by Soskin, Allweiss and Cohen (1932). These authors found that it was possible to obtain perfectly normal blood sugar time curves in the completely depancreatized animal, providing the blood sugar was kept normal by simultaneous administration of glucose and insulin, and *providing the liver was not disturbed.* The findings fit in with more recent observations of Soskin *et al.* (1935) with respect to the relationship between the liver and carbohydrate metabolism in toxæmia.

Application of the knowledge gained from animal experimentation to clinical work is, however, very difficult for a number of reasons; anatomical integrity and functional efficiency are not necessarily synonymous and, as yet, clinical tests of liver function are very unsatisfactory. The reserve capacity of this organ, its ability to regenerate, and the fact that failure of one function does not necessarily imply failure of another are some of the many difficulties in the interpretation of data. However, from a study of many thousands of tests in these laboratories, it would appear that, of all of the methods, those based upon the metabolism of bile-pigments are the most satisfactory; and the two *earliest* signs of impairment of liver function are accumulation of excess quantities of bilirubin in the blood and excretion of excess quantities of urobilinogen in urine.

The writer (1926) drew attention to the high incidence of hyperbilirubinæmia found amongst diabetics; of a group of 130 cases, excess quantities of bilirubin were found in 34 individuals—an incidence of 26·1 p.c. In a later study of a much larger number of cases in this clinic, Dr. A. C. Corcoran (unpublished data) found practically the same incidence; of 500 individuals, normal amounts of bilirubin—less than 0·5 units, that is, 0·2 mg. per 100 c.c. of blood—were found in 301; in 91 cases the amounts ranged between 0·5 and 0·9 units; in

65 cases between 1·0 and 1·4 units ; in 19 cases between 1·5 and 1·9 units, and 24 individuals had 2 units or more. Of the 108 whose bloods contained one or more units of bilirubin, a possible explanation, other than the diabetes (cholecystitis, heart failure, etc.), was found in 34 cases only. In other words, of these 500 individuals, unexplained hyperbilirubinæmia of more than one unit was found in 74 cases—an incidence of 14·8 p.c. It will be noted that the incidence of hyperbilirubinæmia would have been much greater had it been assumed that the bilirubin content of normal blood ranges between 0·2 and 0·5 units only. Assuming that, in the absence of biliary obstruction or hæmolysis, excess quantities of bilirubin in the blood are due to hepatitis, these data are further suggestive of the high incidence of disease of the liver in diabetes mellitus. Suggestive also are the findings with respect to urinary excretions of urobilinogen.

Urobilinogen is a normal constituent of human urine, but the amounts are small. With the technique which they have described, Wallace and Diamond (1925), using Ehrlich's aldehyde reaction, found the amounts were generally such that the pigment was barely detected when the reagent was added to urines which were diluted with 9 parts of water (1 in 10 dilution). Very few urines gave reactions when they were diluted with more than 19 parts of water (1 in 20 dilution). The reactions were negative in 72 of 75 students examined in the morning, and in 48 of 50 students examined in the afternoon. Marcussen and Hansen (1918) reported somewhat similar experiences in normal individuals with the fluorescence method for detection of urobilinogen.

Detectable quantities of urobilinogen in 1 in 20 dilutions of urine are also uncommon in disease. In a routine study of 1000 ambulatory patients, Wallace and Diamond (1925) found such amounts in 10 p.c. of the cases only. That many of these excess quantities were also due to disturbance of liver function is suggested from the conditions in which they were found. The latter included disease of the gastro-intestinal tract ; disease of the biliary tract ; heart failure and disturbance of the hæmopoietic system. These findings fit in with the many thousands of experiences with this test in the metabolism laboratories of the Montreal General Hospital. Combining all of the data, therefore, it appears to be a reasonable assumption that, in the vast majority of cases, when urobilinogen is detected in urine by the above-mentioned test with dilutions greater than 1 in 20, the excess quantities are due to disease.

In the accompanying table are recorded 3000 analyses of bilirubin contents of bloods and 3000 analyses of urobilinogen contents of urines obtained amongst diabetics in the Clinic for Diabetes at the Montreal General Hospital. In all of these examinations, jaundice and cases of suspected jaundice were excluded. It will be observed that if the normal bilirubin content of blood ranges between 0·2 and 0·5 units, excess quantities of bilirubin were found in 27·4 p.c. of these examinations.

The writer (1926) found excess quantities of urobilinogen in the urines of 6 diabetics in a series of 50 cases. It will be noted, however, that the incidence was much higher in the much larger group of cases reported here. Assuming that the normal content of urobilinogen in urine is such that none is detected with dilutions greater than 1 in 20, excess quantities of urobilinogen were found in 27·5 p.c.

TABLE.—*Bilirubin Contents of Blood and Urobilinogen Contents of Urine amongst Diabetics in the Clinic for Diabetes at the Montreal General Hospital.*

(3000 analyses : Cases of jaundice and of suspected jaundice excluded.)

Bilirubin.		Urobilinogen.	
Units.	Number.	Dilution.	Number.
—0·5	2178	Absent	0
0·6	256	—1 : 10	1375
0·7	132	1 : 16	457
0·8	121	1 : 20	342
0·9	81	1 : 25	182
1·0	109	1 : 33	123
1·1	26	1 : 50	171
1·2	19	1 : 100	90
1·3	8	1 : 125	82
1·4	7	1 : 150	63
1·5	16	1 : 166	8
1·6	2	1 : 200	19
1·7	1	1 : 250	42
1·8	6	1 : 333	24
1·9	1	1 : 500	8
2·0	9	1 : 1000	12
2·1	8	1 : 1000+	2
2·2	3	..	..
2·3	4	..	..
2·4	2	..	..
2·5	11	..	..

The remarkable agreement of percentage between urobilinogen and bilirubin is accidental, for, in many cases, though there was hyperbilirubinæmia, the urine contained normal quantities of urobilinogen, and in many cases, though there were excess quantities of urobilinogen in the urine, the bilirubin contents of the bloods were normal.

SUMMARY.

Attention is drawn to the high incidence of hyperbilirubinæmia and excess quantities of urobilinogen in the urines of diabetics. Assuming that, in the absence of biliary obstruction or hæmolysis, excess quantities of bilirubin in the blood are due to hepatic disease, and that excess quantities of urobilinogen in urines are due to disease in the great majority of cases, the above data afford further proof of the high incidence of disease of the liver in diabetes.

REFERENCES.

MARCUSSEN, S., AND HANSEN, S.—(1918) *J. biol. Chem.*, **36**, 381.
MEAD, R.—(1748) Cited by Shields Warren, 'The pathology of diabetes mellitus', (Lea & Febiger), 1930.
RABINOWITCH, I. M.—(1926) *Brit. J. exp. Path.*, **7**, 155.
SOSKIN, S., ALLWEISS, M. D., AND COHEN, D. J.—(1932) *Am. J. Physiol.*, **109**, 155.
SOSKIN, S., ALLWEISS, M. D., AND MIRSKY, I. A.—(1935) *Arch. intern. Med.*, **56**, 927.
WALLACE, G. B., AND DIAMOND, J. S.—(1925) *Ibid.*, **35**, 698.

EXPERIMENTS ON THE IMMUNIZING PROPERTIES OF UNDILUTED AND DILUTED DIPHTHERIA FORMOL-TOXOID.

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SOME months ago Hartley (1935) published the results of experiments on the influence of dilution with normal salt solution on the immunizing power of diphtheria formol-toxoid. His conclusions were that four weeks after one injection of formol-toxoid, guinea-pigs which had been immunized with a non-diluted antigen showed a strikingly lower degree of immunity than those which had received an equal amount of the same toxoid, tenfold diluted with salt solution. Hartley tested the animals for immunity by application of the multiple Schick test dose method (Glenny, Hopkins and Pope, 1924).

In two series of experiments we have investigated the same problem. The first series was carried out during the winter of 1933. The guinea-pigs were immunized by three injections. After a fixed time their immunity was tested by injecting subcutaneously a certain amount of diphtheria toxin. In the second series, carried out recently, the animals received one immunizing injection. The immunity was tested after different intervals by means of the multiple Schick test method.

EXPERIMENTAL.

First Series (1933).

Methods.—Immunization by 3 weekly injections. Immunity tested by injecting 20 minimal lethal doses. Four experiments were carried out (A, B, C and D).

A. Toxoid No. V, 12 flocculation units per c.c.; 3 injections of 6, 12 and 12 flocc. units of undiluted and 5-times diluted toxoid, the total amount of fluid injected thus being 2·5 c.c. and 12·5 c.c. respectively. Immunity test 4 weeks after the last injection.

B. Toxoid No. IX, 16·5 flocc. units per c.c.; 3 injections of 8·25, 16·5 and 16·5 units of undiluted and 5-times diluted toxoid, the total amount of fluid injected being 2·5 c.c. and 12·5 c.c. respectively. Immunity test 4 weeks after the last injection.

C. Original toxoid Ramon, 12 flocc. units per c.c.; 3 injections of 6, 12 and 12 units of undiluted and 5-times diluted toxoid, the total amount of fluid injected being 2·5 c.c. and 12·5 c.c. respectively. Immunity test 4 weeks after the last injection.

D. Toxoid No. IX, same amounts as in B. Immunity test 6 weeks after the last injection.

Results.—The table shows that the diluted toxoid has produced a greater degree of immunity than the undiluted toxoid. In all four experiments the percentage of surviving animals treated with the diluted toxoid considerably surpasses that of the parallel groups.

It is also shown in the last column of the table that the percentage of animals in which the injection of the toxin is not followed by necrosis is greater after the administration of diluted than of undiluted toxoid—a further proof of the higher immunizing power of the former.

TABLE.

	Toxoid.	Number of guinea-pigs.	Surviving.	P.c.	No necrosis.	P.c.
A.	Undiluted	39	29	74	16	41
	Diluted	28	27	96	20	71
B.	Undiluted	43	17	44	5	11
	Diluted	40	27	68	16	40
C.	Undiluted	43	29	67	17	58
	Diluted	34	28	82	22	79
D.	Undiluted	36	18	50	4	11
	Diluted	28	24	86	10	36

Second Series (1935).

Methods.—200 guinea-pigs were immunized with 6·5 flocc. units of toxoid No. XII, containing 13 units per c.c. Half of the animals received this quantity undiluted, the other half in a tenfold dilution with salt solution, *i.e.* in 5 c.c. The degree of immunity was tested after 1, 2, 3, 4, 6, 8 and 12 weeks respectively by means of the multiple Schick test dose technique, the doses of toxin injected in the skin of each animal being 1, 2, 5, 10, 25 and 50 Schick test doses. All groups consisted approximately of the same number of animals (13–15).

Results.—Fig. 1 gives the average number of Schick test doses which could be injected into each animal without producing a positive Schick test.

It must be remembered that the differences between the successive doses

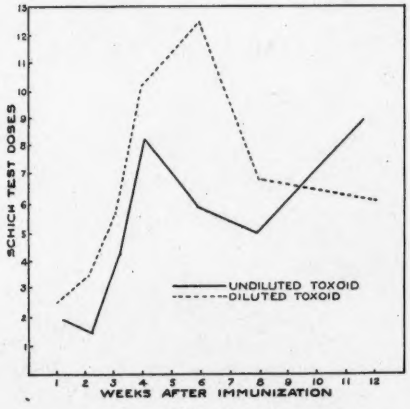

FIG. 1.

of toxin are large, and reactions to intermediate doses could not be detected. Nevertheless the graph shows that up to 8 weeks after immunization the degree of immunity of the animals treated with diluted toxoid is higher than of those treated with the undiluted toxoid.

It is remarkable, however, that after 12 weeks the situation appears to be reversed; the lines have crossed and the immunity of the "undiluted" group is greater. It is to be regretted that we were not in a position to continue this experiment, the stock of immunized guinea-pigs being exhausted. We therefore do not know whether this phenomenon is purely accidental or not. However, the following is perhaps an indication that this reversal is not caused by the imperfection of the method.

In Figs. 2 and 3 are plotted the average Schick-negativeness (*vide supra*) and the percentages of guinea-pigs surviving the injections of the toxin (93 Schick test doses, *i.e.* nearly 2 m.l.d.). Fig. 2 gives the graphs after "undiluted", Fig. 3 after "diluted" immunization. The lines of Fig. 2

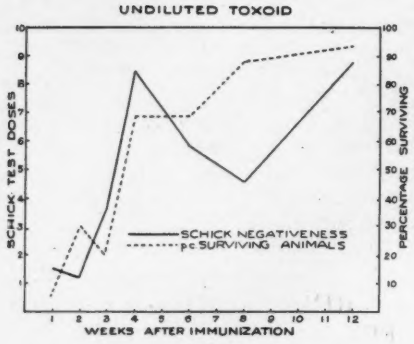

FIG. 2.

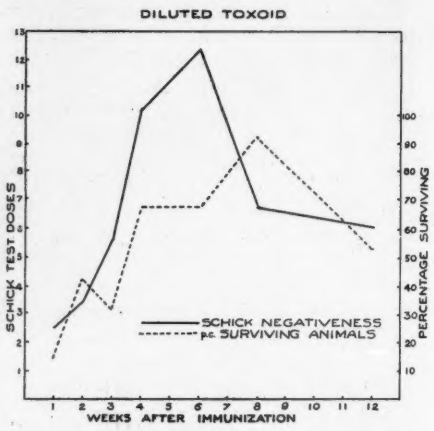

FIG. 3.

do not yet show a tendency to fall after 12 weeks, whereas those of Fig. 3 appear to reach their apices much earlier (after 6–8 weeks). We do not wish to draw definite conclusions from these differences. Further experiments must decide whether they are real or accidental.

DISCUSSION.

Comparison of our results with those of Hartley shows a considerable agreement. In all our experiments we have found that 4 weeks after immunization with diluted toxoid the degree of immunity was higher than after immunization with undiluted toxoid, both after one and after a course of three injections. The results of our second series—one immunizing injection—differ from Hartley's in that the differences in immunity were much more striking in his experiments than in our own. Also, none of his animals, immunized with diluted toxoid, died after the administration of the toxin, whereas in our corresponding experiment (*i.e.* after 4 weeks) 5 out of 15 guinea-pigs died. All our experiments were carried out during the cold season. In spite of the

fact that green cabbage is fed regularly, the immunizability of our guinea-pigs is always lower during this season. This may be the cause of the lower degree of immunity of our animals.

The question whether after a longer period the situation alters is still open. To solve this problem it would be necessary to repeat our experiments on a larger scale.

SUMMARY.

1. The immunizing power of a fixed quantity of a certain diphtheria formol-toxoid is dependent on the total amount of fluid in which it is injected into the animals.

2. During a period up till 8 weeks after immunization, the animals injected with undiluted toxoid are less immune than those immunized with diluted toxoid.

3. Our experiments make it doubtful whether this condition remains unaltered at a later period after the immunization.

REFERENCES.

GLENNY, A. T., HOPKINS, B. E., AND POPE, C. G.—(1924) *J. Path. Bact.*, **27**, 261.
HARTLEY, P.—(1935) *Brit. J. exp. Path.*, **16**, 468.

STUDIES ON THE EXCRETION OF URINARY PORPHYRIN IN RHEUMATIC FEVER.*

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As early as 1880 MacMunn noticed that a pigment belonging to the porphyrin group could frequently be recovered from the urine of patients with rheumatic fever. Garrod (1892–1896) confirmed and extended MacMunn's observation. Since that time an extensive literature has accumulated on the occurrence of porphyrinuria in various diseases; but our knowledge of the relation of porphyrin excretion to acute rheumatism has not advanced beyond the point at which Garrod left it. The present investigation was undertaken to determine the type of porphyrin excreted in acute rheumatism, and to study the relationship of porphyrinuria to activity of the rheumatic process. Our attention was confined to urinary porphyrins, which were detected and identified by spectroscopic examination of ether extracts.

* The work reported in this communication was carried out under the W. K. Kellogg Foundation.

PROCEDURE.

Collection and extraction.—The amount of urine used for each estimation was 1/5 of a 24-hour specimen, or 2/5 of a 12-hour specimen. The porphyrin was extracted by shaking the urine with ether after acidifying with acetic acid. To 10 volumes of urine were added 1 volume of glacial acetic acid and 3 volumes of ether. The mixture was shaken thoroughly in a separatory funnel, and allowed to stand until a definite line of demarcation had formed between the aqueous layer and the ether emulsion. The urine was drained off, and the emulsion broken by shaking it with a few ml. of ethyl alcohol (95 p.c.). The aqueous layer which now separated was drained off and the remaining clear ether layer was freed of acetic acid by washing repeatedly with small volumes of ammonia, at first normal, then 0·1 *N*, until the acidity of the washings had been reduced to pH 4·8. Ammonia is more effective than distilled water in removing not only acetic acid, but also urobilin, which was present in the large majority of extracts, sometimes in such large quantities as to overshadow the porphyrin. There seemed to be no appreciable loss of porphyrin through this procedure, provided that the volume of ammonia used for each washing was small (about 1/10 the volume of the ether), and provided that the pH of each washing after thorough shaking with the ether extract did not exceed 4·8. The washed extract was then adjusted to a standard volume of 16 ml.

Spectroscopic examination.—The extract was placed in a 200-mm. polariscope tube, and examined with a Bausch & Lomb wave-length spectrometer. A ribbon filament projection lamp with a condenser served as light source. The positions (limits and maxima) of individual absorption bands were recorded. It soon became possible to sort out the bands which belonged together, and to interpret the complex absorption spectra observed with reference to Schumm's (1927) data on the spectral characteristics of various urinary pigments.

TYPES OF PORPHYRIN EXTRACTED.

Free porphyrin.—Practically all of the extracts examined contained a pigment with a four-banded spectrum in the visible region characteristic of coproporphyrin, with maxima at the following positions: I, 624 mμ.; II, 568 mμ.; III, 526 mμ.; IV, 495 mμ. The solubilities and absorption spectra of this pigment in various solvents agreed with the data of Schumm (1927) and Fischer (1927) for coproporphyrin. Further proof of its identity was obtained from the "brom-spectral" test described by Schumm, which distinguishes coproporphyrin from other ether-soluble porphyrins with somewhat similar spectra.

In order to determine, if possible, by melting-point which of the four possible isomers of coproporphyrin we were dealing with, coproporphyrin methyl ester was prepared in crystalline form by the following procedure:

The extracts from a large series (approximately 250) of urine samples from rheumatic patients were pooled. This crude material was purified as described by Schumm, by repeated transfers from ether to 5 p.c. HCl and back to ether with the aid of sodium acetate. Following the third transfer to ether an extra step was introduced. The ether solution was washed nearly free of excess acetic acid, then shaken with an equal volume of normal ammonia, which took up the

porphyrin in alkaline solution, leaving a considerable amount of impurity in the ether. From ammonia the material was transferred back to ether by acidifying with glacial acetic acid, thence, after washing, into 25 p.c. HCl. This strong acid solution was washed with alcohol-free chloroform (Schumm), and the pigment transferred back to ether with sodium acetate; the ether solution was washed with 0·1 *N* ammonia, dried with anhydrous Na_2SO_4, and evaporated to dryness. The residue was taken up in about 0·5 ml. of pyridine, to which was added about 5 ml. of absolute ether. This solution was methylated by brief treatment with a solution of diazomethane in absolute ether. The mixture was washed with H_2O to remove excess diazomethane, dried with anhydrous Na_2SO_4, evaporated down to small volume and allowed to crystallize. The amount of crystals finally obtained was very small, probably something less than 2 mg.

Two separate melting-point determinations were made with the aid of a dissecting binocular mounted in a horizontal position. The results were as follows:

(1) Crystals in extreme tip of tube fused at 167° C. Crystals further up shrunk together at 179° C.

(2) First few granules fused at 161° C., a few more at 168° C. They continued to shrink together up to 182° C., where the whole mass fused.

There was too little material for recrystallization.

The melting-points were not sufficiently sharp to be conclusive, especially in the absence of recrystallization. However, information of this sort on porphyrin from biological sources is so scant in the literature and the material itself so tedious to obtain that it seems desirable to present these data. The melting-points for the four synthetic isomers of coproporphyrin methyl ester (as reported by Fischer, Platz and Morgenroth, 1929) are as follows: I, 251° C.; II, 287° C.; III, 145°, also 172° C. Either modification can be obtained from the other by recrystallization; mixtures of the two melt at one point or the other without depression. IV, 168–9° C. The melting-points which we obtained, crude as they are, agree best with either the high-melting modification of isomer III or isomer IV. Since isomer IV is not known to occur in biological material, it is reasonable to conclude tentatively that we are dealing with coproporphyrin III.

Combined porphyrins.—Two compounds of coproporphyrin also occurred in some of the urinary extracts. One of these, which appeared only rarely and in traces, had the absorption bands and solubilities of the copper compound of coproporphyrin. The spectrum in ether comprised two bands in the visible region, with maxima at (I) 561 mμ. and (II) 523 mμ., band I being considerably more intense than band II. Since coproporphyrin combines very easily with copper, and since small amounts of copper are normally present in urine (Rabinowitch, 1933), as well as in laboratory reagents and containers, there is no reason for attaching any physiological significance to the occasional presence of Cu coproporphyrin in urinary extracts.

A second compound, as yet unidentified, occurred much more frequently than the first, and in larger quantities. It showed a two-banded spectrum, easily distinguishable from that of the copper compound. It was found regularly in extracts prepared during the first few months of this study, frequently to the exclusion of free coproporphyrin, and especially in specimens which had been stored for some length of time before extraction. It was less abundant, or absent, in extracts prepared immediately after the close of the collection period. On the hypothesis that this material might be a secondary

product due to some factor in the collection procedure, the use of preservative (formalin) was dispensed with; brown specimen bottles were used, specially cleaned and rinsed with distilled water; urines were protected from direct sunlight, and were extracted as fresh as possible; filtration of sediment from urines was discontinued. Following these refinements in method, most of the 12-hour and many of the 24-hour specimens were free of this porphyrin derivative. In spite of precautions, however, it appeared consistently in the urinary extracts of patients with acute rheumatism.

This substance was stable both in untreated urines and in ether extracts. In the former it increased in quantity with time at the expense of free porphyrin. Its spectrum is of the hæmochromogen type, consisting in ether of two absorption bands in the visible region: I, 581–565 mμ., maximum 575 mμ., approximately; II, 547–529 mμ., maximum 539–40 mμ., approximately. There is another band bordering on the ultra-violet, with a maximum at 405 mμ., but its position is beyond the range of visual measurement. Dilution seems to shift the maxima slightly, as readings of 578 (I) and 541 (II) were frequently obtained on extracts showing very weak absorption. When viewed with a prism spectroscope, band I appears more intense than band II; however, densitometric tracings of a photograph of the spectrum showed that the reverse is actually the case (see Fig. 1, curve B). The spectrum of this substance in aqueous solution, either acid (acetic) or alkaline (NH_3), looks substantially the same as that in ether, except that in alkaline solution band II appears more intense than band I, even to the eye.

As to its chemical properties, the substance is not destroyed by 10 p.c. acetic acid, and from such a solution can be readily extracted by ether. Ether solutions can be shaken without appreciable loss with aqueous acetate solutions between pH 2·0 and 5·0. As the pH is increased above 5·0 (approximately) the substance goes over more and more completely into the aqueous medium. Ether solutions can also be shaken with 0·5 p.c. HCl without loss; but on shaking with 5 p.c. HCl the two-banded spectrum of the complex porphyrin disappears rapidly and completely from the ether, and a different two-banded spectrum, that of free coproporphyrin in acid, appears in the HCl (I, 591 mμ.; II, 549 mμ.). If this acid solution, after removal of the porphyrin-free supernatant ether, is then neutralized with Na acetate and shaken with fresh ether, the typical four-banded spectrum of free coproporphyrin appears in the ether. The unknown substance therefore contains coproporphyrin.

Two-banded spectra in urine and urinary extracts, associated with four-banded spectra of free porphyrin, have been reported by several previous investigators. The positions of these bands, in so far as they can be judged from the published data, correspond very closely with those of our substance. They are distinctly not those of the copper compound. In Table I are summarized the observations of various investigators, including our own, on two-banded pigments from urine and certain other sources.

Stokvis and Garrod both noted that exposure of the two-banded pigment to strong mineral acids changed the spectrum considerably (giving the bands of free porphyrin in acid at 591 and 550 mμ. approximately); and that subsequent neutralization yielded, not the original pigment, but the four-banded spectrum of free porphyrin in alkaline solution. These authors also found

TABLE I.—*Summary of Data on Two-banded Porphyrin Pigments.*

Nature of material.	Approximate positions of maxima of absorption bands. I. (mμ.)	II. (mμ.)	Author.
Pathological urine (? diagnosis) extract of precipitate in neutral aqueous solution	576	542	Stokvis, 1889.
Same pigment after removal of accompanying four-banded pigment	578	540	„ „
Four-banded pigment from same urine, treated with $ZnCl_2$ and NH_3	578	540	„ „
Urine (lead poisoning); extract of soda precipitate treated with NH_3 for several days	577	541	Garrod, 1892.
Pink urate sediments, amyl alcoholic extract	578	542	„ „
4-banded urinary pigment + $ZnCl_2$ and NH_3	578	540·5	„ „
Weakly alkaline solution of uroporphyrin after 24 hours' incubation at 39° C.*	576	538	Günther, 1922.
Urine (sulphonal porphyria)	577	542	Schumm, 1927 (p. 155).
Culture filtrates of *C. diphtheriæ*	574	537	Coulter and Stone, 1931.
Mass cultures of *C. diphtheriæ*, *B. phosphorescens* and yeast; acetic-ether extracts	574	537	Stone and Coulter, 1932.
Urine (acute rheumatism):			
Ether extract	575	538	Kapp and Coburn.
In higher dilution	578	541	„ „
Coproporphyrin + $ZnCl_2$ and NH_3	574·5	538·5	Schumm, 1916.
Porphin phyllin in pyridine-ether	575·4	543	Fischer and Gleim, 1935.
Etiophyllin in pyridine-ether	581·2	541·9	Fischer, Filser & Plötz, 1932.

* This spectrum has been shown by Derrien and Benoit (1928) to be due to the zinc compound of uroporphyrin, formed as a result of prolonged contact of the solution with Jena glass.

that the two-banded spectrum could be very well reproduced by treating the four-banded urinary pigment (presumably coproporphyrin) with $ZnCl_2$ and NH_3. It may be pertinent to note that the zinc compound of uroporphyrin was found to be the predominant form of urinary porphyrin in a fatal case of acute porphyrinuria studied by Derrien and his collaborators (1928, 1930).

Zinc is not, however, the only possibility. Garrod suggested that his pigment might be a calcium or magnesium compound, but did not succeed in preparing these substances artificially for comparison. Quite recently the magnesium compounds (so-called phyllins) of a number of porphyrins, mostly in the plant series, have been prepared in Fischer's laboratory. They are all labile to acids in varying degree, and the spectra of the simpler members of the series (especially porphin and etioporphyrin) are quite similar to those of our compound (Fischer, Filser and Plötz, 1932; Fischer and Gleim, 1935). Whether our substance is a compound of zinc or magnesium or some other metal is now under investigation. Complete spectrographic characterization will be reported at a later date.

Densitometric tracings* of the visible absorption spectra of crude urinary extracts are shown in Figs. 1 and 2. The curves have been oriented so as to be comparable with absorption curves as they are customarily represented. The maxima of the curves correspond to maxima of absorption in the extracts. Curve A in Fig. 1 represents the absorption of light by an extract containing a small amount of free coproporphyrin. Curve B represents the absorption spectrum of another extract containing combined porphyrin alone. Curve C (Fig. 2) was obtained from a third extract in which both free and combined coproporphyrin were present. This curve results from the summation of two spectra, similar to A and B, one upon the other. The two bands of the porphyrin compound show as "humps" on the sides of bands II and III of free porphyrin. The steep end-absorption of curve B, in which all wave-lengths shorter than about 500 mμ. are completely absorbed, is due to urobilin. Purified preparations of combined porphyrin are completely transparent in this region.

FIG. 1.

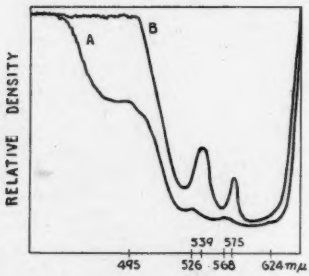

FIG. 2.

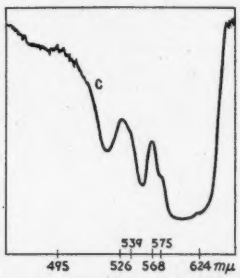

FIG. 1.—Absorption curves of crude urinary extracts containing (A) free coproporphyrin, (B) combined coproporphyrin.

FIG. 2.—Absorption curve of crude urinary extract containing both free and combined coproporphyrin.

The extracts from which curves A and C were obtained appeared to be free of urobilin, their absorption maxima at about 495 mμ. being due to band IV of free coproporphyrin. Their opacity at wave-lengths shorter than about 485 mμ. was due to yellow impurities, not to the porphyrin itself. The apparent end-absorption in all three curves, beginning at about 650 mμ., is an artefact caused by the limit of sensitivity of the photographic plate on which the original spectrograms were recorded.

GENERAL OBSERVATIONS ON THE EXCRETION OF COPROPORPHYRIN.

In the course of the first year of this work (1932–1933), urine specimens of about 150 patients in various stages of health and disease were examined for the presence of porphyrin. From these observations there emerged several general indications. They are the following: (1) The method was sufficiently sensitive to permit the detection of porphyrin in the urine of normal persons in good health. (2) Quiescent rheumatic subjects in good health excreted

* The spectrum plates were made with a Hilger E1 spectrograph, with a photoflood lamp as source; and the tracings were made with a Kraus recording densitometer.

porphyrin in quantity similar to that found in other healthy persons. (3) Adults usually excreted larger amounts of porphyrin than children. (4) In 41 cases of acute rheumatism, the amount of urinary porphyrin was definitely above normal, and the intensity of the porphyrinuria was strikingly greater in severe attacks than in mild ones. This finding substantiates that of Garrod (1892). In our series several sudden fatalities which occurred unexpectedly were preceded by intense porphyrinuria. (5) The excretion of abnormally large amounts of porphyrin in acute rheumatism bore no relation to fever, hæmaturia, or the use of therapeutic measures *per se*. (6) In rheumatic patients with oliguria or *in extremis*, porphyrin could no longer be detected in the urine. It reappeared in large amounts when renal function was re-established. (7) In five rheumatic subjects with febrile diseases other than rheumatism (influenza and pneumococcus pneumonia), the amount of urinary porphyrin was within normal limits.

SERIAL STUDIES.

As this study progressed, it was found that single readings, or even readings taken at intervals, were difficult to interpret because of the wide variations in output from day to day, especially during active disease. Consecutive daily observations over considerable periods were therefore undertaken on a limited number of individuals. In the absence of a quantitative method of estimating the amount of porphyrin found, the observations were recorded in a semi-quantitative way, the amount of porphyrin being expressed in terms of an arbitrary unit.* These studies included several groups of subjects: (1) normal persons in good health; (2) patients with acute rheumatism; (3) patients with hæmolytic streptococcus throat infections, specifically pharyngitis and scarlet fever.

Normal persons in good health.—The daily porphyrin output of normal healthy subjects ranged between 1 and 4 units. According to Fikentscher (1932) and Schreus and Carrié (1933), the actual amount of porphyrin excreted by such persons varies from 20 to 80 γ per day. If these figures are correct, then our unit probably represents a quantity of the order of 20 γ; and since our estimations were carried out on only 1/5 of the total output of urine, the actual quantity present in an extract reading 1 unit must have been of the order of 4 γ, *i. e.* 0·004 mg.

We also confirmed the findings of Schreus and Carrié that there are

* *Definition of unit.*—As already mentioned, the same fraction of the daily output of urine was extracted in each case, and the extracts were examined under standard conditions of depth (200 mm.) and illumination. The "unit" was that intensity of absorption under these standard conditions which just permitted the position of the coproporphyrin band in the red to be determined with certainty. This region was chosen because it was least subject to interference from strong end-absorptions due to yellow pigments (especially urobilin) which were usually present in the extracts.

Intensities of 5 units were checked in two ways: (1) At this level of concentration, a relatively faint band of the coproporphyrin spectrum at about 598 mμ. was just measurable, using the standard 200-mm. cell. (2) The same extract, examined in a column 1/5 of the standard length—40 mm. instead of 200—gave a reading of 1 unit. Intermediate intensities, between 1 and 5 units, were estimated, and readings above 5 units were obtained by the multiplication of estimations made in the 40-mm. cell. It is apparent that this procedure involved a large personal factor, and was far from strictly quantitative. However, the same observer (E. M. K.) performed all of the readings, and could repeat them days or weeks apart within 1 unit in either direction. Changes of 2 units and over can be taken as significant for the present discussion.

considerable differences between individuals with respect to their characteristic levels of urinary porphyrin. This fact is of some importance in the interpretation of porphyrinuria in disease, as the normal level of the patient has to be taken into account. Thus a reading of four units may be practically normal for one patient, yet may mean a fourfold increase in porphyrin excretion for another.

Patients with acute rheumatism.—The findings in four typical patients with acute rheumatism are charted in Fig. 3. During periods of acute symptoms, all excreted amounts of porphyrin far above the normal range. It will be

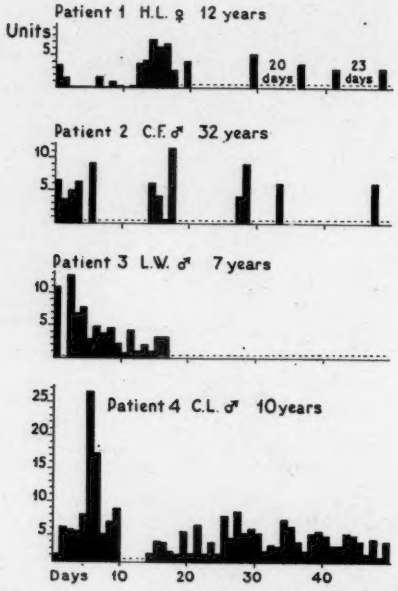

FIG. 3.—The daily urinary porphyrin of 4 patients with acute rheumatic fever. ▪ Total coproporphyrin, both free and combined. — Urine not examined.

seen that the high level was not maintained continuously, but was subject to fluctuation from day to day and from week to week.

In a few patients it was possible to study the excretion of porphyrin before, as well as during, an attack of acute rheumatism. In Fig. 4 are shown records of three patients who were kept under observation from the onset of hæmolytic streptococcus pharyngitis through a symptom-free interval into the period of an acute attack.

These three records show that the increase in porphyrin excretion accompanied or followed shortly after the onset of rheumatic symptoms. The attacks were less severe than those of the patients of Fig. 3, and the intensity of porphyrinuria developed was correspondingly lower. However, all developed at least a fourfold increase over the level present at the start of the observation period.

Patients with hæmolytic streptococcus throat infections.—In view of the close association between infection of the respiratory tract with hæmolytic streptococcus and the development of an attack of rheumatic fever, a second group of patients was studied to determine whether a similar increase in urinary porphyrin occurs following infections with this organism in the absence of rheumatism. Two clinical types of infection were selected for this study—simple pharyngitis and scarlet fever.

Records of the daily porphyrin excretion of five patients with pharyngitis are charted in Fig. 5. None of these patients had ever had rheumatic fever, and convalescence was uncomplicated in all cases. There was no consistent increase of porphyrin excretion in the group, either during the infection or subsequently. With the possible exception of Patient 6, who showed a slight initial rise, their porphyrin excretion was essentially normal.

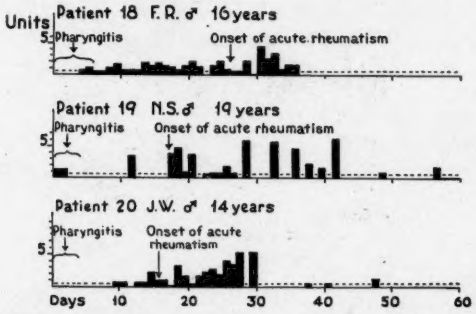

FIG. 4.—The daily urinary porphyrin of 3 patients previous to and during an attack of acute rheumatism. ▪ Total coproporphyrin, both free and combined. — Urine not examined.

Similar findings were obtained in rheumatic subjects with hæmolytic streptococcus pharyngitis, when the throat infection was not followed by a rheumatic recrudescence. The urinary porphyrin in such cases showed no increase whatever over periods of five weeks from the onset of pharyngitis.

The records of five patients with scarlet fever are shown in Fig. 6. One of these patients (17) had had rheumatic fever as a child, the others had not. Convalescence was uncomplicated in all instances. All but one in this group showed a definite increase in urinary porphyrin during the first few days of their infection. Patient 16, who had severe scarlatina, maintained a fairly high level throughout the period of observation. In Patient 17 there was a marked tendency for the amount of porphyrin excreted to rise and fall in cycles of 10 to 12 days. But in no case did the porphyrin show an increase during convalescence like that found following the onset of a rheumatic attack.

It seems, then, that the marked increase of porphyrin which accompanies acute rheumatism is not merely a late result of the preceding respiratory infection, but is a phase of rheumatic disease as such.

In contrast to free porphyrin, which could be detected in almost every specimen examined, the two-banded porphyrin compound discussed earlier in this paper was consistently absent from the urine of certain patients. It

occurred in the urines of most of the patients with acute pharyngitis, but disappeared permanently with the subsidence of symptoms. In the scarlet

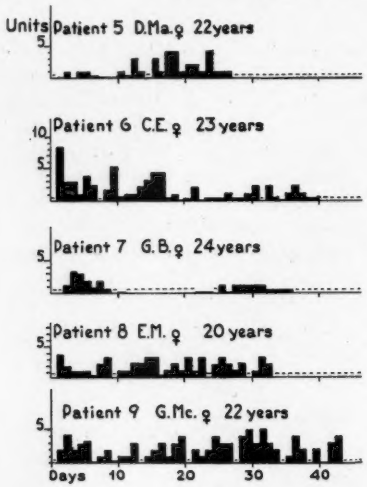

FIG. 5.—The daily urinary porphyrin of 5 patients with hæmolytic streptococcus pharyngitis. ▪ Total coproporphyrin, both free and combined. — Urine not examined.

fever group the three milder cases were consistently free of it ; but it appeared irregularly in Patients 16 and 17 during convalescence. In acute rheumatism,

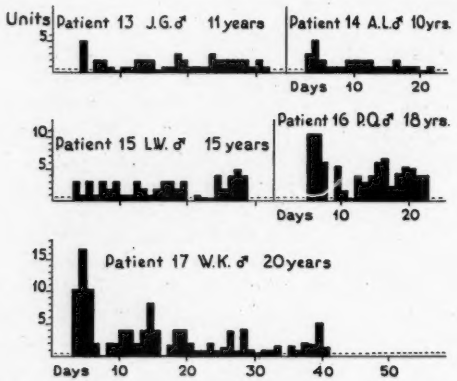

FIG. 6.—The daily urinary porphyrin of 5 patients with scarlet fever. ▪ Total coproporphyrin both free and combined. — Urine not examined.

however, it has been consistently present in the urine of every patient examined. The formation of this compound is partly a function of time, and appears to depend on the presence of some chemical factor (substance or condition) which

occurs in some urines and not in others. What that factor is, and whether it is of any significance for rheumatic disease, it is not yet possible to say.

DISCUSSION.

The observations just recorded show that there is a definite relationship between porphyrinuria and acute rheumatism. No explanation of this relationship is brought out by the data. However, the findings of other investigators seem to throw some light on the possible origin of porphyrinuria.

Garrod (1893), in discussing the significance of porphyrins in human disease, observed that many patients with porphyrinuria, irrespective of the nature of their diseases, showed evidence of liver damage. In the recent literature (*i. e.* work done since Fischer's clarification of the chemistry and isomerism of the porphyrins), the concept is frequently advanced that an increase in the amount of porphyrin excreted in the urine signifies a derangement of liver function (Althausen, 1931; Thiel, 1934; Schmidt and Herold, 1934; Carrié and Herold, 1935; Fikentscher, 1935; Francke and Fikentscher, 1935; Watson, 1935).

A different though possibly related view of the origin of coproporphyrinuria has been expressed by Schreus and Carrié (1933). They attribute the increase in urinary coproporphyrin which accompanies fever (from various causes) to the increased catabolism of blood-pigment, since they were able to show that erythrocyte destruction was also increased during the febrile period. This is a reasonable hypothesis, at least for cases where the urinary porphyrin has the same spatial configuration as hæmin—that is when it is derived from etioporphyrin III (see Schreus, 1934).

In view of the above considerations, the porphyrinuria of acute rheumatism is in line with clinical experience. The hæmorrhagic character of the disease (Coburn, 1933) and the accompanying anæmia both imply blood-pigment destruction. Moreover, there is abundant evidence of liver damage in cases coming to necropsy (Ball, 1886; Poynton and Schlesinger, 1931; Coburn, 1933); and the findings of Chadwick (1928), based on a lævulose tolerance test, point to disordered hepatic function in cases of moderate severity.

Since both blood destruction and liver damage accompany activity of the rheumatic process, and since either may be expected to give rise to an increase in urinary porphyrin, the findings presented here cannot be attributed to either factor alone. However, since the liver is involved in the normal removal of blood-pigment from circulation as well as in porphyrin metabolism, the porphyrinuria of rheumatism can be interpreted as an expression of disturbed liver function. An explanation of the exact nature of the disturbance must wait for a fuller understanding of normal porphyrin metabolism.

SUMMARY.

(1) The urine of patients with rheumatic fever contains abnormally large amounts of coproporphyrin, qualitatively indistinguishable by visual spectrometric methods from the porphyrin normally present in the urine of healthy persons.

(2) Coproporphyrin isolated from the urine of rheumatic patients has been provisionally identified as isomer III.

(3) The urine of rheumatic patients nearly always contains part of the coproporphyrin in combination, presumably with a metal. This combined porphyrin has been observed only sporadically in urines from other sources.

(4) The increase of urinary porphyrin appears concurrently with the symptoms of rheumatism. There is no increase following recovery from hæmolytic streptococcus throat infections in the absence of rheumatic attack.

(5) The porphyrinuria of acute rheumatism is therefore related to the rheumatic process itself and not to the preceding respiratory infection.

(6) The possible significance of these observations is discussed.

We are indebted to Dr. C. B. Coulter for assistance and advice throughout this investigation ; to Dr. Hugh H. Darby of the Bartol Research Foundation for the spectrographic records ; and to Dr. Emma Dietz of Harvard University for advice in connection with the preparation of porphyrin esters.

REFERENCES.

ALTHAUSEN, T. L.—(1931) *Klin. Wschr.*, **10**, 1016.
BALL, B.—(1886) " Du rheumatisme visceral," *Thèse de Paris.*
CARRIÉ, C., AND HEROLD, L.—(1935) *Klin. Wschr.*, **14**, 196.
CHADWICK, R. T.—(1928) *Arch. Dis. Child.*, **3**, 179.
COBURN, A. F.—(1931) ' The factor of infection in the rheumatic state '. Williams & Wilkins, Baltimore.
Idem.—(1933) *Amer. J. Dis. Child.*, **45**, 933.
COULTER, C. B., AND STONE, F. M.—(1931) *J. gen. Physiol.*, **14**, 583.
DERRIEN, E., AND BENOIT, CH.—(1928) *Bull. Soc. Chim. Fr.*, **43**, 522.
DERRIEN, E., AND CRISTOL, P.—(1930) *C.R. Soc. Biol.*, Paris, **103**, 126.
FIKENTSCHER, R.—(1932) *Biochem. Z.*, **249**, 259.—(1935) *Z. Geburtsh. Gynäk.*, **111**, 164.
FISCHER, H.—(1927) *Ber. dtsch. chem. Ges.*, **60**, 2611.
Idem AND DUESBERG, R.—(1932) *Arch. exp. Path. Pharmak.*, **166**, 95.
FISCHER, H., FILSER, L., AND PLÖTZ, E.—(1932) *Liebigs Ann.*, **495**, 1.
FISCHER, H., AND GLEIM, W.—(1935) *Ibid.*, **521**, 157.
FISCHER, H., PLATZ, K. AND MORGENROTH, K.—(1929) *Hoppe-Seyl. Z.*, **182**, 265.
FRANCKE, K., AND FIKENTSCHER, R.—(1935) *Münch. med. Wschr.*, **82**, 171.
GARROD, A. E.—(1892) *J. Physiol.*, **13**, 598.—(1893) *J. Path. Bact.*, **1**, 187.—(1894) *J. Physiol.*, **15**, 108.
Idem AND HOPKINS, F. G.—(1896) *J. Path. Bact.*, **3**, 434.
GÜNTHER, H.—(1922) *Ergebn. allg. Path. path. Anat.*, **20**, 608.
MACMUNN, C. A.—(1880) *Proc. Roy. Soc.*, **31**, 206.—(1883) *Ibid.*, **35**, 370.
MILROY, J. A.—(1918) *Biochem. J.*, **12**, 318.
POYNTON, F. J., AND SCHLESINGER, B.—(1931) ' Recent advances in the study of rheumatism.' Blakiston's Son & Co., Inc., Philadelphia.
RABINOWITCH, I. M.—(1933) *J. biol. Chem.*, **100**, 479.
SCHMIDT, H. R., AND HEROLD, L.—(1934) *Arch. Gynäk.*, **156**, 463.
SCHREUS, H. TH., AND CARRIÉ, C.—(1933) *Klin. Wschr.*, **12**, 745.
SCHREUS, H. TH.—(1934) *Ibid.*, **13**, 121.
SCHUMM, O.—(1927) ' Spektrochemische Analyze ' (2nd ed.). Gustav Fischer, Jena.
STOKVIS, B. J.—(1889) *Ned. Tijdschr. Geneesk.*, **13**, No. 2, 413.
STONE, F. M., AND COULTER, C. B.—(1932) *J. gen. Physiol.*, **15**, 629.
THIEL, W.—(1934) *Klin. Wschr.*, **13**, 700.
WATSON, C. J.—(1935) *J. clin. Invest.*, **14**, 106.

THE ESTIMATION OF PHOSPHATASE IN FLUORIDE BLOOD.

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THE estimation of phosphatase in fluoride blood is not so simple as it would appear to be at first sight. Numerous reasons can be brought forward to support this statement, but probably the most important is the great divergency of opinion in regard to the accuracy of the methods already published. The inhibitory effect of fluoride on the urease reaction with urea is so well known as to need but a casual reference, but it is not definitely established whether this inhibition holds good for all enzymes, although it is generally assumed to be so.

Fluoride is generally used in blood as an anticoagulant and preservative, and in the majority of cases it answers this purpose remarkably well. In fact it had been shown by Roe, Irish and Boyd (1927) that 10 mg. of sodium fluoride per c.c. of blood, collected with sterile precautions, will preserve the blood for ten days for the estimation of non-protein nitrogen, uric acid, creatinine, sugar and cholesterol. This is probably due to the prevention of enzyme activity by the fluoride, but as to whether fluoride also acts as an enzyme preservative no information is available.

In a recent paper by one of us—Rose (1933)—it was shown that if the fluoride was removed by suitable means urea could still be estimated by urease methods. This suggested that if the same method was used to remove the fluoride, estimation of the enzyme activity within the blood might be possible. On investigating the methods available for the estimation of phosphatase we arrived at the conclusion that the method of Bodansky (1932–33) offered the best possibilities. This method takes less time than that of Kay (1930), and in general gives much more accurate and consistent results.

The first stage is the estimation of the inorganic phosphorus in the blood, and in the main we found that our results agreed with those of White and Monaghan (1933). Unfortunately these investigators did not state whether the blood was allowed to stand for any length of time before analysis. As Table I shows, considerable differences can occur if the blood is allowed to stand in an unpreserved condition; on the other hand the presence of fluoride appears to keep the inorganic phosphorus constant. The figures given in Table I seem to us to suggest that fluoride prevents enzyme activity, and in consequence that a more accurate estimation can be obtained if the inorganic phosphorus is estimated in fluoride blood. Further investigation on the estimation of the enzyme itself seemed to support this assumption.

On reference to Table II it will be seen that fluoride has a marked inhibitory effect on the phosphatase activity even in comparatively small concentration.

TABLE I.

Specimen No.	Time in hours.	Inorganic phosphorus mg./100 c.c. Serum.	Potassium oxalate 10 mg./c.c.	Sodium fluoride 10 mg./c.c.
1	1	4·30	4·26	4·12
1	24	4·56	4·30	4·12
1	72	4·84	4·40	4·13
2	1	2·90	2·84	2·84
2	24	3·26	3·00	2·86
2	72	4·68	3·22	2·88

TABLE II.

Specimen No.	Phosphatase in Bodansky units. Serum.	Potassium oxalate 10 mg./c.c.	Sodium fluoride 10 mg./c.c.
1	4·84	4·80	1·91
2	4·22	3·75	0·52
3	3·05	2·96	1·39
4	15·01	14·96	3·02

Before endeavouring to remove the fluoride by means of caffeine magnesium salicylate, it was found to be an essential that the latter substance should be prepared in strict accordance with the method described by Rose (1933).

To estimate the phosphatase in fluoride blood, 25 mg. of caffeine magnesium salicylate were thoroughly mixed with 1 c.c. of the blood and allowed to stand for a few minutes. The estimation was then carried out on this blood in the ordinary way. Using this technique, we found that the results on the caffeined blood tended to be high, but this was probably due to the fact that in the majority of cases the resultant colour when developed was slightly turbid. To overcome the turbidity an equal volume of absolute alcohol was added, which has the property of dissolving up the cloud and giving a clear bright

TABLE III.

Specimen No.	Time in hours.	Phosphatase in Bodansky units. Serum.	Potassium oxalate 10 mg./c.c.	Sodium fluoride 10 mg./c.c.	Sodium fluoride 10 mg./c.c. plus 25 mg. caffeine magnesium salicylate/c.c. immediately before analysis.
1	1	4·84	4·80	1·91	4·92
1	24	4·52	4·46	1·02	4·90
1	72	3·86	3·92	1·02	4·84
1	96	2·92	3·01	1·06	3·52
4	1	15·01	14·96	3·02	15·34
4	24	12·06	12·22	3·00	15·30
4	72	9·62	10·61	2·62	15·20
4	96	9·58	9·62	2·60	12·06

solution. When the latter was compared with the standard and calculation made to take into account the deviation from Beer's law, as stated in the paper of Bodansky (1933), much more accurate results were obtained.

The figures given in Table III show quite clearly that an accurate estimation of phosphatase can be made up to a period of 3 days if the blood is collected into fluoride, and that the fluoride acts as a preservative to the phosphatase normally present in blood.

SUMMARY.

1. The inhibitory effect of fluoride on phosphatase has been shown.
2. A method has been indicated for the estimation of phosphatase in the presence of fluoride.

REFERENCES.

Bodansky, A.—(1932) *J. biol. Chem.*, **99**, 107.—(1933) *Ibid.*, **101**, 93.
Kay, H. D.—(1930) *Ibid.*, **89**, 235.
Roe, J. H., Irish, O. J., and Boyd, J. I.—(1927) *Ibid.*, **75**, 685.
Rose, C. F. M.—(1933) *Brit. J. exp. Path.*, **14**, 339.
White, H. L., and Monaghan, B.—(1933) *Proc. Soc. exp. Biol., N.Y.*, **31**, 1.

SOME CHEMICAL METHODS FOR THE DETECTION AND ROUGH ESTIMATION OF AGAR IN BIOLOGICAL MATERIALS.

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Specific soluble substances and other extractives are often made from bacteria that have been grown on agar; it is therefore possible that these products are in some cases contaminated with agar, and that this is responsible for the observed carbohydrate reactions. Three methods are described in this paper for the recognition of agar in the presence of various other naturally occurring substances, and by a judicious combination of these three it should be possible to identify agar with certainty under most conditions. The only substance which has been found that would be confused with agar under all conditions is the polysaccharide from carragheen moss; like agar this is made from sea-weed.

Some space is devoted to the consideration of the chemical implications of these colour tests. The ordinary derivatives of sugars with a five- or six-membered oxygen-containing ring do not give the actions, but derivatives of sugars in the open chain or aldehydo form resemble agar fairly closely.

There is a large group of sugar tests which depend essentially on the decomposition of the sugar with a hot acid and the coupling of the decomposition products with some cyclic compound, so that a strongly-coloured derivative

is made. These sugar tests vary in sensitivity and in specificity according to the conditions of the decomposition and to the nature of the cyclic substance. Under certain conditions the action goes with such regularity that it may be used as a method of estimation, and Tillmans and Philippi (1929) have used the orcin and sulphuric acid test of Tollens in this way. Dische (1929) has studied some of these reactions, and he (1930) has used colour reactions with carbazol and with diphenylamine for the estimation of thymus nucleic acid. In this paper the technique is described for using these three tests for the recognition and rough estimation of agar in the presence of the commonly occurring sugars.

THE ORCIN REACTION.

Sørensen and Haugaard (1933) have used a modification of the Tillmans-Philippi reaction for the recognition of the individual sugars in a mixture such as a polysaccharide or protein. Bell and Robinson (1934) have made use of the reaction in this way. The extinction co-efficient of the solution is measured for a number of different wave-lengths after varying times of heating, and the resulting curves are interpreted in the light of the known behaviour of the individual sugars. The extinction co-efficient of a mixture was found to be the sum of the extinction co-efficients of its components. Sørensen and Haugaard give figures for the colour development with a number of sugars, and they comment on the redness of the galactose colour.

This observation suggested the use of the test for the recognition of agar, for I had noticed, while using the Tillmans-Philippi method of carbohydrate estimation, that agar, unlike other carbohydrates, gave an initial red colour which later turned to the standard orange brown. The conditions of the test have been modified slightly, and a Lovibond tintometer is used instead of the more elaborate Stufenphotometer for measuring the colours.

At a temperature of 55° C. a colour that is identical with the red of the tintometer scale is developed when 1 ml. of a solution of galactose or agar is mixed with 10 ml. of 66 p.c. sulphuric acid (1198 g. of concentrated acid mixed with 340 g. of water and kept cool) containing 0·2 p.c. of orcin. It is necessary to adhere strictly to this temperature and to this acid concentration, or the colour will be either orange or purple. At a lower temperature the colour comes up more slowly, but the rates of colour development with different carbohydrates bear the same ratio to one another that they bear at 55° C. At higher temperatures the action proceeds inconveniently fast and there is more yellow in the final colour.

A colorimeter may be used for the matching if a colour standard is used. Such a standard, which matches the tintometer red exactly, is got with methyl red in phthalate buffer at pH 4·8. This solution is, however, rather too clear and needs dilution with a complementary colour ; a suitable mixture consists of 30 ml. of 0·002 p.c. methyl red in $M/5$ buffer, 1·1 ml. of 0·016 p.c. bromocresol green and 0·4 ml. of 0·016 p.c. bromocresol purple. This solution in a 1 cm. cell has a colour corresponding to 11–11·5 red units (R.U.).

Galactose and agar under the conditions defined give much the same amount of colour in 30 minutes ; the mean of a number of measurements is 18 red units for 0·25 mg. with a probable error of 1 R.U.

In each case the intensity of the colour is proportional to the amount of carbohydrate present, and each is affected similarly by variations in the concentration of the acid, *e. g.* :

0·25 mg. agar	1·25 ml.	H_2O	9·75 ml.	H_2SO_4	give	15	R.U.
,,	1·00	,,	10·00	,,	,,	17·3	,,
,,	0·75	,,	10·25	,,	,,	19·4	,,
,,	0·50	,,	10·50	,,	,,	20·8	,,

The results of a number of experiments with carbohydrates are recorded in Table I ; a number of experiments with hexitols, pentitols and non-reducing sugar acids which give no colour, and with fructose and its derivatives, *e. g.* sucrose, melezitose, raffinose, lupeose and glucosone, which give a strong yellow colour almost immediately, are not included. In Table II agar is compared with a number of galactose derivatives. Agar is clearly distinguishable from the substances in Table I by the fact that in those cases where the colour is developed rapidly it is always very much yellower than that due to agar. The substances in Table II on the other hand develop the colour more slowly. This rapid development of a red colour is the basis of a method for the approximate estimation of agar. The method cannot be used with material containing fructose pentoses or uronic acids in addition to agar, for the yellow colour produced by the former masks the red of the latter.

There is substantial unanimity among the workers on the subject that agar contains 25–35 p.c. of galactose (Koch, 1887 ; König and Bettles, 1905 ; Lüdtke, 1929 ; Uyede, 1929). This figure has been arrived at by mucic acid estimation, by precipitation of the galactose with methylphenyl hydrazine and by isolation. I have got a similar figure from fermentation experiments with galactose-trained yeast. There is therefore little doubt that it is galactose itself that occurs in an agar hydrolysate and not merely a related substance, such as galacturonic acid or galactonic acid.

The behaviour of agar.—The behaviour of agar with this reagent differs in two respects from that of galactose : in the first place the colour is developed more rapidly, and in the second place three to four times as much colour is developed as would have been expected from the galactose content. The peculiar behaviour of agar may be explained in two ways : Firstly, the colour may be due to galactose, and the exceptional behaviour of agar may be due to the mode of linkage of the galactose or to the presence of an unusual isomer of it. Secondly, there may be an unusual sugar in agar. It will be shown that the first explanation is not adequate to account for all the facts.

Sørensen and Haugaard (1933) found that lactose gave a colour equal to that given by a mixture of glucose and galactose. In Table II figures are given for the colour development with a number of different galactose derivatives ; it is clear from this table that behaviour similar to that of agar is not caused by any of these types of linkage. Two of these substances are polysaccharides containing galactose only. Sinistrin or galactogen is found in snails, and was prepared from them by a method based on that of May (1934). Galacto-carolose is a metabolic product of *Penicillium charlesii* (Clutterbuck, Haworth, Raistrick, Smith and Stacey, 1934) ; I am indebted to Prof. Raistrick

TABLE I.

Substance.	Weight (mg.).	Colour reaction. 3 minutes. R.U.	Y.U.	B.U.	6 minutes. R.U.	Y.U.	B.U.	9 minutes. R.U.	Y.U.	B.U.	15 minutes. R.U.	Y.U.	B.U.
Glycogen	0·25	..	..	..	..	..	..	..	..	..	1·1	0·7	0
Yeast gum	0·25	..	..	..	..	..	..	..	..	..	1·1	0·6	0
Yeast gum (galactose trained)	0·25	..	..	..	..	..	..	..	..	..	1·1	0·6	0
Trehalose	0·25	..	..	..	..	..	..	..	..	..	1·8	0·8	0
Diacetone glucose	0·50	..	..	..	..	..	..	..	..	..	2·5	1·6	0
Mannose	0·25	..	..	..	1·1	0·7	0	2·3	1·2	0	5·1	2·2	0
Ovomucoid	2·00	..	..	..	..	..	..	..	..	..	1·1	0·8	0
Serum polysaccharide	1·00	..	..	..	..	..	..	1·1	0·3	0	..	..	..
Thymus nucleic acid	1·50	..	..	..	..	..	..	..	..	..	2·0	0·8	0
Gum tragacanth	0·50	..	..	..	..	..	..	..	..	..	8·0	10·0	0
Menthol glucuronic acid	0·50	..	..	..	..	..	..	1·3	1·2	0	2·4	2·0	0
Turnip pektin	1·50	..	..	..	..	..	..	4·2	5·0	0	..	..	..
Xylose	0·25	2·5	1·0	0·1	5·5	1·6	0	8·2	2·4	0	..	..	..
Arabinose	0·25	2·4	1·0	0	3·5	1·4	0	6·4	2·3	0	15·3	4·0	0
Rhamnose	0·25	..	..	..	..	..	..	3·0	9·0	0	..	..	..
Gum arabic	0·50	3·3	1·5	0	..	..	..	5·0	2·2	0	10·3	3·0	0

In each case the amount of carbohydrate stated is suspended in 1 ml. of water and heated for the time stated at 55° C. with 10 ml. of the acid solution of orcin. The resulting colour is expressed as the sum of the three constituent colours, red, yellow and blue, on the arbitrary scale of the tintometer.

TABLE II.

Substance.	Weight (mg.).	Colour reaction. 3 minutes. R.U.	Y.U.	B.U.	6 minutes. R.U.	Y.U.	B.U.	9 minutes. R.U.	Y.U.	B.U.	15 minutes. R.U.	Y.U.	B.U.
Agar	0·25	3·2	0·1	0·2	5·6	0	0·4	8·5	0	0·6	..	..	..
Galactose	0·25	..	..	..	1·4	0·8	0·1	3·0	0·3	0·1	8·0	0·2	0·2
Penta-acetyl α galactopyranose	0·54	..	..	..	1·8	0	0·1	3·0	0·1	0·2	..	..	..
Penta-acetyl β galactopyranose	0·54	..	..	..	1·6	0	0·1	3·6	0·2	0	5·0	0	0·3
Galactose methylphenyl hydrazone	0·50	..	..	..	..	..	..	3·3	0·7	0·1	..	..	..
Lactose	0·50	..	..	..	1·0	0·4	0·1	2·1	0·6	0·1	7·5	0·1	0·2
Melibiose	0·50	..	..	..	0·7	0	0·2	2·2	0	0·1	7·0	0·1	0·2
Galacto-carolose	0·25	..	..	..	1·2	0	0	2·4	0	0·1	6·0	0	0·1
Sinistrin	0·25	..	..	..	1·1	0	0	2·5	0	0	8·0	0	1·0
Psychosine	1·00	..	..	..	1·0	0·3	0	2·0	0·3	0	6·1	0	0·3
Frog-spawn jelly	1·50	..	..	..	3·5	1·2	0	6·0	1·0	0·2	12·0	1·0	0·3

for the sample used. These two polysaccharides are not identical, for galactocarolose is more rapidly hydrolysed by acids than galactogen; furthermore they have rather widely different optical rotations. The frog-spawn jelly, of which the behaviour is described in Table II, was prepared by treating the spawn for 2–3 hours with 0·7 p.c. potassium cyanide solution and centrifuging out the eggs. The supernatant fluid was precipitated with alcohol and enough acetic acid to make it neutral; this precipitation was repeated several times. The product like that of van Ekenstein and Blanksma (1907), contains 16–20 p.c. of galactose. Yeast gum, whether from ordinary baker's yeast or from galactose-trained yeast (Stephenson and Yudkin, 1936), was prepared by Salkowski's (1894) method. The psychosine used was a sample prepared by Dr. Weil-Malherbe; it had the expected sugar content, and the rate of colour formation is in agreement with Pryde and Humphrey's (1926) conclusion that the galactose is in the pyranose state. Galactose methylphenyl hydrazone in solution in 1 : 1 acetic acid and alcohol gives a colour very similar to that given by galactose if the solution is used immediately. If, however, the solution is kept for some hours or if it is heated, the solute decomposes and a very much more yellow colour develops with the orcin reagent.

For various reasons, some of which have been published in a recent paper (Pirie, 1936*a*), it seemed probable that at least a part of the galactose in agar exists in the open chain or aldehydo form. Derivatives of this type were therefore prepared (Pirie, 1936*b*) and their behaviour is recorded in Table III. It is clear that this change in the mode of linkage of the sugar has elicited properties very similar to those of agar from glucose, galactose and mannose; the pentose derivatives, like the pentoses themselves, give an orange colour. Under the conditions of the test all these derivatives would be rapidly hydrolysed, and with the mercaptals the smell of ethyl mercaptan is recognizable almost immediately. Penta-acetyl galactofuranose gives the colour more rapidly than the pyranose derivatives, but not so rapidly as derivatives of aldehydo galactose. If we assume that the colour is due in all cases to the aldehydo sugar that is present in equilibrium with the other forms, this is in agreement with the observations of other workers; for Brigl *et al.* (1931) have found that galactose has a greater tendency than glucose to exist in the free aldehyde form, and Compton and Wolfrom (1934) find that the furanose ring opens more readily than the pyranose ring. Since the rate of colour-formation varies in this way according to the mode of linkage of the sugar, great caution must be exercised in attempts to use the orcin method as a means of identifying sugars—for example in glucosides or when attached to a protein.

Agar contains only about 30 p.c. of galactose, but the colour developed by it in 6–9 minutes is approximately the same as that developed by the same weight of galactose in the aldehydo form. The colour-formation cannot therefore be explained in terms of the galactose alone, and it is necessary to assume that the other sugar or sugars in agar play some part in it. Very mild acid hydrolysis, *e. g.* boiling for 45 minutes with $N/40$ H_2SO_4, has little or no effect on the colour given by agar, but if the hydrolysis is sufficiently vigorous to liberate any free galactose (estimated by fermentation with galactose-trained yeast or by precipitation with methylphenyl hydrazine), the red colour is no longer developed quickly, but comes up slowly as with galactose

| | | |

TABLE III.

Substance.	Weight (mg.)	Colour reaction. 3 minutes. R.U.	Y.U.	B.U.	6 minutes. R.U.	Y.U.	B.U.	9 minutes. R.U.	Y.U.	B.U.
Agar	0·25	3·2	0·1	0·2	5·6	0	0·4	8·5	0	0·6
Carragheen carbohydrate	0·50	2·1	0	0·1	4·1	0	0·2	6·1	0	0·3
Kanten	0·25	3·3	0·2	0	5·0	0	0·3	7·6	0	0·5
Penta-acetyl galactofuranose	0·54	..	..	..	2·4	0	0·2	4·0	0	0·2
Penta-acetyl aldehydo galactose	0·54	3·1	0	0·1	5·0	0	0·5	7·2	0	0·7
Penta-acetyl galactose mercaptal	0·63	2·0	0·3	0·1	4·1	0	0·2	6·8	0·9	0
Galactose mercaptal	0·33	2·4	0	0	5·1	0	0	9·3	0	0·2
Hepta-acetyl galactose	0·70	3·4	0	0·3	5·3	0	0·6	7·9	0	0·6
„ glucose	0·70	5·0	0	0·9	8·1	0	1·4	11·0	0	1·5
„ mannose	0·70	4·5	0	0·5	8·0	0	1·0	10·0	0	0·9
Hexa-acetyl arabinose	0·70	4·3	0·3	0·1	5·3	0·8	0	6·2	2·0	0
„ rhamnose	0·66	4·7	1·6	0	6·2	3·6	0	7·3	5·6	0
Penta-acetyl mannose mercaptal	0·72	6·0	0	0·3	10·0	0	0·7	13·0	0	1·1

or mannose. An aldehydo sugar would go back to the ring form under these conditions.

When agar is heated for a short time with dilute acids it loses the property of forming a jelly, and becomes a water-soluble non-diffusible polysaccharide which may be precipitated in the usual way with alcohol, ammonium sulphate and, to some extent, with basic lead acetate. Agar is partially broken down in a similar way when autoclaved; this process has been studied by Takahashi and Shirahama (1934), who called the soluble product "hydrato kanten λ". Commercial samples of agar contain 5–20 p.c. of this soluble non-diffusible material. It can be extracted by freezing a 5–10 p.c. jelly and allowing it to thaw; the fluid is then extracted in a press. If this process is repeated several times it is found that the carbohydrate content of the expressed fluid is high at first, but soon falls, and, since more kantene is produced each time the agar is autoclaved, it keeps constant during many further extractions. It is clear, therefore, that bacteria grown on agar may carry with them a certain amount of this soluble carbohydrate; the amount will, of course, vary with the species and the treatment which the agar has received. This has already been recognized by Sordelli and Mayer (1931), Linton (1933) and Zozaya (1933) as a factor which seriously interferes with the isolation of specific soluble substances from some bacteria grown on agar.

Preparation of kanten.—100 g. of agar is autoclaved with 1500 ml. of water for 15 mins. at 15 lb. per sq. inch. The solution is poured into shallow metal trays, allowed to cool and then put into an ice-making machine till it is completely frozen. The mass is allowed to thaw in a warm part of the room, put in a calico sheet and pressed. The fluid measures 800–900 ml. The process is repeated four times with a litre of water each time. Total carbohydrate estimations by the Tillmans-Philippi method give the following values on successive extracts: 1·1, 0·7, 0·5, 0·5, 0·5 p.c. Agar or kanten give, with the Tillmans-Philippi method, a colour that is indistinguishable both in character

and intensity from that given by the same weight of glucose or galactose. The combined extracts are distilled *in vacuo* to 300 ml., frozen and allowed to thaw. Some gelatinous material, closely resembling agar in its physical properties, is rendered insoluble by this treatment and can be centrifuged off. The fluid is now precipitated with two volumes of alcohol and the solid redissolved and precipitated by the addition of one volume of saturated ammonium sulphate. This precipitate is dialysed and dried. I have found that the technique of drying used already with oxidized glutathione (Pirie, 1931) works very well here. The solution of polysaccharide, containing 0·5–5·0 p.c. of solid, is put in a flat nickel dish and frozen solid; it is then put into a vacuum desiccator containing fresh sulphuric acid and exhausted rapidly. A good water pump will evacuate the dessicator sufficiently to make the rate of absorption of heat by conduction and convection smaller than the rate of loss of heat as latent heat of vaporization, the material, therefore, remains frozen and the ice evaporates directly. By this means a fine friable mass is produced which is very soluble in water. With solutions containing more than 1 p.c. of solids it is advisable to use a nickel dish, for glass dishes are often broken; it is also advisable to freeze the solution before putting it in the desiccator, for if it is allowed to freeze by rapid evaporation a froth frequently forms with a skim on it, and this hinders the evaporation of the ice below.

This material is probably closely related to that which contaminates many bacterial preparations, and its separation from them is a matter of considerable difficulty; it can, however, easily be recognized. Kanten and agar are indistinguishable by the reactions described in this paper.

Estimation of agar.—The total carbohydrate content of the substance is first estimated by a modification of the Tillmans-Philippi method. To 1 ml. of the test solution 10 ml. of 66 p.c. sulphuric acid, made up as before, containing 0·2 p.c. of orcin is added. This mixture is heated on a briskly-boiling water-bath for 10–15 minutes. A standard containing 0·25 mg. of galactose is treated in exactly the same way. The tubes are cooled and diluted to 50 ml. They are again cooled and measured in a colorimeter. The colour is stable for ½–1 hour if kept in the dark; after longer intervals solutions containing agar deposit a brown precipitate. The orcin reagent becomes discoloured at room temperature after a day, but will keep for longer at 0°.

An amount of the substance which will contain 0·25 mg. of total carbohydrate is dissolved in 1 ml. of water and put in a boiling tube in a water-bath at 55° C. 10 ml. of the orcin reagent preheated to 55° are then added and samples are withdrawn and measured on the tintometer after 3, 6 and 9 minutes. If pentoses, fructose, etc., are absent, the colour at 6 minutes is proportional to the agar content with an accuracy of 10–15 p.c.; the colour at 9 minutes serves as a check on this value, but it is less reliable. If less than 15 p.c. of the carbohydrate present in a substance is agar, the test gives very uncertain results.

No absolute specificity can be claimed for this test. It is given by breakdown products of agar, by all the aldehydo sugars tested and, as Table III shows, by a constituent of carragheen moss carbohydrate. It can, however, be taken as evidence of the presence either of a somewhat unusual sugar or else of a sugar in a somewhat unusual state of combination.

THE CARBAZOL REACTION.

Dische (1930) has described a test for thymonucleic acid in which carbazol is used in the presence of sulphuric acid. Under the conditions recommended by Dische no very characteristic reaction is given with agar, but if modified slightly a relatively specific test is obtained. The test depends more on the tint of the colour than on its intensity, and the fluids are not quite clear. It is therefore unsuitable for quantitative work.

To 1 ml. of the aqueous test solution 0·2 ml. of 0·5 p.c. alcoholic carbazol is added and then 9 ml. of the 66 p.c. sulphuric acid already used ; the mixture goes very slightly blue. It is put in a boiling water-bath for 2 minutes. Under these conditions agar and its derivatives, carragheen moss carbohydrate and some sugar mercaptals, *e. g.* galactose and mannose, give a clear blue colour with no trace of violet, mauve or purple. The other carbohydrates that have been tried (all those used with the orcin reaction) and their derivatives either give little or no colour in two minutes or else give mauve or purple colours. The test works well with 0·25 mg. of agar or kanten and about twice that quantity of carragheen carbohydrate ; the latter, however, contains only 60 p.c. of sugar as estimated by the orcin method at 100° C.

Dihydroxyacetone, glyceric aldehyde, methylglyoxal and formaldehyde also give a blue colour with this reagent. The first three can be excluded as the cause of the reaction in the case of agar, since the colour given is not very strong ; with formaldehyde, on the other hand, 0·02 mg. gives as much blue colour as 0·25 mg. of agar. For a number of reasons, however, it is unlikely that formaldehyde is produced during the reaction in any appreciable quantity. Treatment of agar for a few minutes with 66 p.c. sulphuric acid at 100° does not lead to the formation of volatile substances which will react with carbazol, and more prolonged heating gives a distillate which develops a purple colour ; furfuraldehyde gives a very similar colour. A test with a large amount of formaldehyde, *e. g.* 0·1 mg., gives a blue precipitate ; this precipitate will also separate with formaldehyde in the presence of agar, but it will not separate with agar alone, even when large amounts are used. Finally formaldehyde gives no red colour with orcin at 55° C., but only a brown. Of these substances only glyceric aldehyde gives an orcin reaction that resembles in any way that given by agar. A red colour appears quickly in this case, but after 2–3 minutes it goes brown.

Agar, the sugar mercaptals and the carbohydrate derived from carragheen moss by extraction with hot water and freezing and drying the extract, all give about equal amounts of blue colour if quantities containing equal amounts of carbohydrate, as estimated by the use of the orcin reagent at 100° C., are used.

THE DIPHENYLAMINE REACTION.

A 2 p.c. solution of diphenylamine in glacial acetic acid which contains 2·5 volumes p.c. of sulphuric acid has been used by Dische (1930) as a specific test for thymus nucleic acid. When 1 ml. of a sugar-containing solution and 2 ml. of this reagent are heated in a boiling-water bath a blue colour quickly develops with thymus nucleic acid, whereas little or no colour develops with hexoses or pentoses even after 20 minutes. With agar, carragheen

polysaccharides and aldehydo sugars or their acetylated derivatives a strong green colour appears in 10–20 minutes. As in the case of the orcin reaction, the distinction between agar and other carbohydrates is in the rate of development of colour and not in the tint of the colour, but unlike that test, this one can be used in the presence of pentoses and uronic acids. This test in contradistinction to the carbazol test is positive with hepta-acetyl hexoses, hexa-acetyl pentoses and the aldehydo sugars, but fails with the sugar mercaptals. This failure is due to the interference of the mercaptan present; for it is obvious from the smell of mercaptan that the mercaptal is hydrolyzed under the conditions of the test; furthermore, if ethyl mercaptan is added to the mixture for a test with agar or a hepta-acetyl sugar the colour development is impaired.

With this test the trioses and furfuraldehyde, but not formaldehyde, give strongly positive reactions. Fructose gives more colour than the hexoses, but only one-fifth the colour that is given by the same weight of agar or by an amount of carragheen polysaccharide with the same carbohydrate content as estimated by 100° C. orcin measurements.

Besides the blue colour that is given by the carbohydrate of thymus nucleic acid and the green colour that is given by the substances discussed in this paper, this diphenylamine reagent has the property of giving a purple colour with the carbohydrate residue that is attached to certain proteins. The serum proteins, ovomucoid and the mannose and glucosamine-containing polysaccharide isolated from the products of the alkaline hydrolysis of serum proteins by the methods of Levene and Mori (1929) or of Rimington (1931) all give this colour to an extent that is roughly proportional to their carbohydrate content. This reaction is not given by free mannose or glucosamine, but it has not yet been investigated further.

It is possible to use this colour action for the approximate estimation of agar either with a colorimeter or with the tintometer. In 10 minutes 1 mg. of agar gives a colour matched by 6·3 yellow tintometer units and 6·3 blue units; the colour goes on developing if the mixture is heated longer, but in 20 minutes a certain amount of colour is given by other sugars also. The amount of colour is nearly proportional to the amount of agar present, and the proportion of yellow to blue remains constant over a wide range of colour intensity; if kept cold the colour is stable for at least half an hour.

Certain fractions of hydrolysed agar have been found which do not react with this reagent, although they give colours with the other two; the test therefore is slightly less useful as an index of contamination with agar, but this defect is more than offset by the fact that it can be used in the presence of pentoses or uronic acids. The test is inapplicable to solutions which contain any considerable quantity of either nucleic acid or serum proteins.

DISCUSSION.

Three colour reactions are described in this paper which have been developed from some well-known reactions by the use of a lower temperature, a shorter time of heating, or a more dilute acid during the development of the colour. Under these mild conditions the constituents of agar are still able to give a colour, whereas most of the other carbohydrates that have been studied are inert. The derivatives of the aldehydo sugars behave similarly, and it is

possible that the behaviour of both agar and the carbohydrate from carragheen moss is due to the presence in these polysaccharides of sugars in the aldehydo form. The amount of colour that is given by agar makes it probable that it is not only the galactose that is in this state of combination.

Agar and carragheen moss have two constituents in common—galactose and a sulphuric acid ester. It has been pointed out already that the former is insufficient alone to account for the colour formation; the latter is likewise inadequate, for the uncrystallizable part of the acetylation products of agar (Pirie, 1936*a*) gives each action as strongly as the corresponding amount of agar, although it is almost sulphate-free.

These tests are not in a strict sense specific for agar, but they are relatively characteristic of sugars bound in the open chain or aldehydo form, and they may be given by any substance which contains a sugar bound in a similar manner. In so far as the tests are useful the usefulness lies in the apparent rarity of this type of linkage in nature.

SUMMARY.

Three colour reactions have been applied to the detection of agar in the presence of certain other substances. The polysaccharide of carragheen moss and some synthetic derivatives of the aldehydo sugars react similarly.

Two of the tests may be used for the rough estimation of agar.

REFERENCES.

Bell, J. C., and Robinson, C. G.—(1934) *J. chem. Soc.*, 813.
Brigl, P., Mühlschlegel, H., and Schinle, R.—(1931) *Ber. dtsch. chem. Ges.*, **64**, 2921.
Clutterbuck, P. W., Haworth, W. N., Raistrick, H., Smith, G., and Stacey, M.—(1934) *Biochem. J.*, **28**, 94.
Compton, J., and Wolfrom, M. L.—(1934) *J. Amer. chem. Soc.*, **56**, 1157.
Dische, Z.—(1929) *Microchemie*, **7**, 33.—(1930) *Ibid.*, **8**, 9.
van Ekenstein, W. Alberda, and Blanksma, J. J.—(1907) *Chem. Weekbl.*, **4**, 407.
Koch, F.—(1887) *Ber. dtsch. chem. Ges.* (ref), **20**, 145.
König, J., and Bettles, J.—(1905) *Z. Untersuch. Nahr.-u. Genussm.*, **10**, 457.
Levene, P. A., and Mori, T.—(1929) *J. biol. Chem.*, **84**, 49.
Linton, R. W., and Shrivastava, D. L.—(1933) *Proc. Soc. exp. Biol., N.Y.*, **31**, 406.
Lüdtke, M.—(1929) *Biochem. Z.*, **212**, 419.
May, F.—(1934) *Z. Biol.*, **95**, 277.
Pirie, N. W.—(1931) *Biochem. J.*, **25**, 619.—(1936*a*) *Ibid.*, **30**, 369.—(1936*b*) *Ibid.*, **30**, 374.
Pryde, J., and Humphreys, R. W.—(1926) *Ibid.*, **20**, 825.
Rimington, C.—(1931) *Ibid.*, **25**, 1062.
Salkowski, E.—(1894) *Ber. dtsch. chem. Ges.*, **27**, 497.
Sordelli, A., and Mayer, E.—(1931) *C.R. Soc. Biol.*, Paris, **107**, 736.—(1931) *Ibid.*, **108**, 675.
Sørensen, M., and Haugaard, G.—(1933) *Biochem. Z.*, **260**, 247.
Stephenson, M., and Yudkin, J.—(1936) *Biochem. J.*, **30**, 506.
Takahashi, E., and Shirahama, K.—(1934) *J. Fac. Agric. Hokkaido Univ.*, **35**, 101.
Tillmans, J., and Philippi, K.—(1929) *Biochem. Z.*, **215**, 36.
Uyeda, Y.—(1929) *J. Soc. Chem. Ind. Japan*, **32**, 568.
Zozaya, J., and Medina, L.—(1933) *J. exp. Med.*, **57**, 41.

STAPHYLOCOCCAL TOXOID IN THE TREATMENT OF DIABETES.

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GILCHRIST and Wilson (1934) propounded the theory that the most important ætiological factor in diabetes was infection by staphylococci. When no overt infection was present, as was usually the case, they believed that staphylococci could almost always be isolated from the accessory nasal sinuses, and that treatment of this sinusitis would result in amelioration of the diabetic condition. Acting on this opinion, they treated diabetics with staphylococcal toxoid, and claimed that all the patients showed improvement in their clinical condition, often considerable and sometimes dramatic. Daily injections of insulin were in most cases replaced by weekly injections of toxoid, and all the cases showed lowering of insulin requirements. In addition to this there was a pronounced improvement in the patient's condition, as judged by a number of rather doubtful criteria. The advantages claimed were so important that at the suggestion of Dr. R. D. Lawrence we decided to give the treatment a thorough trial.

First, the concentrations of naturally occurring staphylococcal antitoxin in the sera of diabetics and normal individuals were compared. Estimations were made on 52 diabetics taken at random from the large diabetic clinic at King's College Hospital. These corresponded quite closely with the normal figures given by Murray (1935), and the results are shown in Tables I and II.

TABLE I.—*The Antitoxin Titre in 52 Diabetics.*

Units of antitoxin	<0·4	0·4	0·7	1·0	1·5	2·0	3·0
Diabetics p.c.	18·0	31·0	15·0	8·0	16·0	4·0	8·0

TABLE II.—*A Comparison of Normal and Diabetic Cases.*

Units of antitoxin	0·7 or less	1·0	1·5 or over	Average
Diabetics p.c.	64·0	8·0	28·0	0·94 units
Murray's normals p.c.	67·0	17·0	16·0	0·84 ,,

The estimations were performed by the technique described by H. J. Parish *et al.* (1934), and the results are expressed in international units of staphylococcal antitoxin.

Of these 52 cases, 29 were selected for treatment and observation. Well-known cases of all types and ages were taken, their choice being governed by their reliability and the possibility of their attendance for the necessary weekly injection. The severity of the diabetes varied from cases on no insulin, to one on 100 units of insulin a day. They were all under close observation as out-patients and their condition was judged by urine and blood-sugar tests, frequently repeated throughout the trial period and for some months after.

The patients were given four doses of toxoid (·05, ·1, ·2, ·4 c.c.) at weekly intervals. Reactions to the injections were usually slight, only a few patients complained and only one patient had a really sore arm. After this course the antitoxin estimations were repeated, and then 17 of the patients were given four more weekly doses (·6, ·8, 1·0, 1·0 c.c.). These larger doses did not give any unpleasant reactions, being tolerated even better than the earlier doses. After this the antitoxin was re-estimated, and then again between four and six months after the completion of the course. Although the average initial antitoxin was almost the same in the diabetics and in Murray's normal cases, the increase in titre following administration of toxoid was slightly less in our diabetic cases. The fall after a few months as shown by Murray's cases also occurred in ours.

TABLE III.—*Antitoxin Response of Diabetics to Toxoid.*

	Antitoxin titre.								Average.
	1·5.	2.	3.	5.	8.	11.	17.	26.	
Toxoid ·75 c.c.	4	16	32	20	16	0	8	4	6·02
,, 4·15 c.c.	0	10	42	14	21	7	3	3	5·9
4–6 months after finish of course	21	49	10	14	3	3	0	0	2·9

TABLE IV.—*Response Compared to Normal.*

		Antitoxin titre.				Average.
		1·5 and less.	2.	3–8.	11–38.	
Diabetics p.c.	Before	88	4	8	0	0·94
	After	0	10	77	13	6·02
Murray's normals p.c.	Before	89	10	1	0	0·84
	After	2	13	58	27	7·50

The diabetic condition of these 29 patients kept remarkably constant throughout and for some months following the injections, as is shown in Table V, the insulin requirements in most cases being unchanged. Overt staphylococcal lesions are quite uncommon among well-controlled diabetics, and thus it was not possible to form an accurate idea of the value of the toxoid in these infections. One case who had had boils for years cleared up rapidly after commencement of the treatment, but another case of septic foot did not respond until insulin was started. Many of the patients said that they felt much better after a few injections had been given, but these feelings of well-being were not substantiated by changes in their diabetic condition.

TABLE V.—*Results of Treatment.*

On insulin		26 cases.
	Insulin decreased	2 cases.
	,, increased	3 ,,
	,, unaltered	21 ,,
No insulin		3 cases.
	Insulin started during treatment	2 cases.
	,, ,, 10 weeks after	1 case.
	Remark.—No improvement with toxoid but improvement at once with insulin.	
Sepsis		4 cases.
	At beginning of treatment	2 cases.
	During treatment	1 case.
	10 weeks after treatment	1 ,,

SUMMARY.

The naturally occurring staphylococcal antitoxin is equal in diabetics and normals.

Diabetes is not influenced by the injection of staphylococcal toxoid, although the same increase in titre of the antitoxin is produced in diabetics as in normals.

Staphylococcal lesions were not sufficiently common among the diabetics for a trial of their treatment by toxoid to be made.

We wish to thank Dr. R. D. Lawrence for his critical interest in the investigation, which he suggested, and Dr. E. ff. Creed for technical advice. We are indebted to Messrs. Burroughs Wellcome for a supply of toxoid and reagents for the estimations.

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REFERENCES.

GILCHRIST, J. A., AND WILSON, M. J.—(1934) *Can. med. Ass. J.*, **30**, 353.
MURRAY, D. S.—(1935) *Lancet*, **1**, 303.
PARRISH, H. J., O'MEARA, R. A. Q., AND CLARK, W. H. M.—(1934) *Ibid.*, **1**, 1054.

INFLUENZA VIRUS ON THE DEVELOPING EGG: I. CHANGES ASSOCIATED WITH THE DEVELOPMENT OF AN EGG-PASSAGE STRAIN OF VIRUS.

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A BRIEF account has already been published (Burnet, 1935*a*) of the isolation of a strain of the virus of epidemic influenza from an Australian epidemic. In all essential respects this strain appears to be identical with the human strains isolated in England (Smith, Andrewes and Laidlaw, 1933) and America (Francis, 1935). Serologically it is either identical or very closely related to the W.S. strain, since the horse-serum IH2 produced by immunization with W.S. inactivates the Australian strain to a high titre. Serum from a ferret convalescent from an infection with our strain was sent to Hampstead and found to inactivate the W.S. strain (Andrewes, private communication). There was a slight discrepancy in the relative activities of the ferret serum and IH2, the former appearing to be more active against the Australian strain, while IH2 was more active in the test made at Hampstead with W.S. virus. However, in each case the apparently weaker serum had travelled across the tropics by the ordinary mail, and may well have deteriorated to some extent on the way. The symptoms and lesions produced in ferrets and mice are entirely similar to those described for the English strain.

As soon as the strain had been established for ferrets and mice, attempts to propagate it on the developing egg were commenced. Insignificant lesions were at first obtained, but the virus was undoubtedly multiplying, as typical infections in ferrets and mice could be obtained with egg-membrane emulsions. Precisely similar findings have recently been described by Wilson Smith (1935). Although the early lesions on the egg were relatively trivial, a certain proportion of membranes showed tiny proliferative foci differing slightly in appearance from the non-specific granular opacities which are fairly frequent in uninfected eggs. This encouraged us to continue passage for a large number of generations, with the result that the virus has steadily increased in its pathogenicity for the egg. The strain is now so active that relatively large, easily counted specific foci are produced after 2 days' incubation, and the embryo is killed with characteristic lesions by the 3rd or 4th days. It is the object of this paper to describe the changes in the activity of the virus during the course of this development of pathogenicity for the egg.

METHODS.

Ferrets were inoculated by instilling about 0·25 c.c. of material into the nares under light ether anæsthesia. For passage experiments they were killed during the secondary rise of temperature

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usually 5 or 6 days after inoculation, and the contents of the nasal cavity scraped out to provide material for further inoculation. Rectal temperatures were taken twice daily.

Mice were inoculated under anæsthesia by dropping about 0·05 c.c. of fluid on to the external nares and allowing the mice to draw in the liquid. In our experience a chloroform-ether mixture was more satisfactory than ether alone in producing rapid even respiration and allowing more uniform inoculations. The mice were killed and their lungs examined 5 days after inoculation. The extent of the lung lesions was recorded on suitable diagrams and numerically evaluated 0, 1, 2, 3, or 4, according to the scheme used at Hampstead. Slight degrees of œdema and congestion were disregarded, the figures referring to the extent of the areas of plum-coloured consolidation only.

Eggs were inoculated according to the technique used in a number of previous investigations (Burnet, 1933, 1936). The eggs were first incubated at 38·5° C., but after experience with ectromelia virus had indicated the beneficial effect of a lower temperature, 36° was used as the routine temperature. Passages were made every 3 days as a rule, but 2- or 4-day periods were fairly often interposed, if more convenient. Up to the present the virus has not been kept outside the egg for more than a few hours. Spare stocks are kept in 50 p.c. glycerol Ringer solution at −4° C. and preserve their activity well for at least 3 months, but no recourse has been made to these for the main passage series.

All subinoculations from egg membrane lesions have been made by grinding up the whole of the membrane forming the base of the artificial airspace with 5 c.c. of broth. The emulsion was centrifuged and the supernatant fluid used for passage or for animal inoculation.

BEHAVIOUR OF FERRET- AND MOUSE-PASSAGE STRAINS.

In order to provide a basis for comparison with the egg-passage strain a brief account will be given of the behaviour of the Australian virus in ferret and mouse passage.

Only a small series of ferret passages was made, the strain being lost when lung passage was attempted. Table I shows the first 6 passages. Its chief interest is the evidence it provides for the sudden appearance of mouse pathogenicity at the 4th passage. The necessity for from 2 to 13 ferret passages before a human strain can be adapted to mice has been reported by Andrewes, Laidlaw and Smith (1935), and provides another indication of the similarity of the Australian strain to English ones. (Table I.)

TABLE I.—*Ferret Passage of Influenza Virus.*

Ferret No.	Passage.	Inoculum.	Maximum temperature.	Result of inoculation in mice.
F2	1	Human nasal washings.	105·2° F.	0, 0, 0, 0, 0, 0, 0, 0
F3	2	F2 M.F.	106·0° F.	..
F4	3	F3 E.	105·2° F.	1, 0, 0, 0, 0, 0, 0, 0, 0
F8	4	F4 E.	106·3° F.	0, 2, 2, 3
F10	5	F8 E.	105·8° F.	0, 2, 3, 4
F12	6	F10 M.F.	106·1° F.	..

Notes.—E. = emulsion of ground-up nasal mucosa of ferret shown. M.F. = Elford membrane filtrate (A.P.D. 0·8μ) prepared from emulsion of nasal mucosa. Results of mouse inoculation expressed according to the method used by Laidlaw, Smith, Andrewes and Dunkin (1935).

Mouse passage was initiated by inoculating with an emulsion of the nasal mucosa of Ferret 8 (4th passage). No difficulty has been experienced in continuing passage either with emulsions of pneumonic lung or filtrates made

from this with gradocol membranes of average pore diameter approximately 0·8μ. Certain fluctuations in virulence appeared during the first 20 passages, but for some months the virus has been killing most of the mice inoculated with undiluted emulsion or filtrate in 3 to 5 days.

DEVELOPMENT OF PATHOGENICITY FOR THE EGG.

An account has already been published of the first 15 passages of influenza virus on the egg (Burnet, 1935*b*). The series was initiated with a membrane filtrate from Ferret 10, which was at the same time inoculated intranasally into a ferret and 4 mice. The ferret F12 showed typical temperature rise and symptoms, while the mouse results were recorded as 0, 2, 3, 4. Of the 8 eggs inoculated, 5 appeared normal, 3 showed slight focal opacities suggesting weak specific lesions. By the 15th passage practically all eggs were showing recognizable lesions in the form of small opaque foci scattered irregularly over the membrane. Very few of these foci exceeded 0·2 mm. in diameter, and some of the infected membranes could not with certainty be distinguished from an uninfected membrane showing unusually marked non-specific lesions. At this stage a membrane emulsion produced typical influenza in a ferret and was weakly active in mice, 2 out of the 4 inoculated showing definite mild lesions.

At the 23rd passage the ferret inoculated from the membrane showed only a single temperature rise to 104·1° F., with no secondary rise. Mice, however, on this occasion suffered more extensive lesions than usual. At about this time a test was made to compare incubation at 37° and 39° C. Judging from a small number of membranes the 39° lesions appeared to be more easily recognized than those incubated at 36°–37°, mainly because non-specific reactions were less at the higher temperature. Passage was continued at 39° with a slow increase in the size and distinctness of the specific foci. A second comparison of 36°–37° with 39° at the 35th passage was decidedly in favour of the lower temperature, and since then passage has been at 36°–37°.

There was a fairly rapid further improvement in the foci, and by the 46th passage serial dilutions gave rise to focal lesions in number approximately proportional to the amount of virus used. Most eggs were opened 3 days after inoculation, but in each of the 48th and 50th generations, 2 eggs when opened on the 5th day showed a living embryo and retrogressing membrane lesions. Deaths of the embryo clearly due to infection by the virus were first noted at the 52nd passage when 2 were dead when the eggs were opened on the 4th day. Well-marked membrane lesions were present, and the embryos were obviously only recently dead. The only abnormality noticed was extreme congestion of the abdominal viscera.

Between the 52nd and 62nd generations most eggs which were opened on the 4th day after incubation at 36° were dead. At 39° C. they survived at least 6 days.

At the 63rd passage a new phenomenon made its appearance. Three eggs opened on the 3rd day all contained dead embryos showing what appeared to be an acute hæmorrhagic encephalitis. The vault of the skull was bulging abnormally with a dark purplish mass showing through the tissues. On

opening the skull the brain was found almost disintegrated, with very numerous hæmorrhages into the ventricles and throughout its substance. The intensity of the pathogenic action of the virus on the embryo continued to show a progressive increase, and by the 71st passage practically all eggs were dead within 48 hours of inoculation. The macroscopic appearances showed a significant change, the hæmorrhages into the tissues being no longer confined to the brain. A few still showed the massive hæmorrhagic swelling of the brain, but in most dead embryos multiple hæmorrhages into the feather follicles and in the substance of the muscles were equally prominent. The appearance of the embryo was indeed very similar to that observed after infection with Newcastle disease virus, but the membrane lesions were still typically influenzal. The early death of the embryo prevented their development to the extent of those of about the 60th generation, but they were well defined and readily counted.

TABLE II.—*Summary of Egg Passages with Australian Influenza Virus.*

Egg passage.	Result of subinoculation to—		Notes on egg lesions.
	Ferrets.	Mice.	
1	..	0, 1, 3, 4	3 eggs slight lesions, 5 *nil.*
2	106·1°+	1, 2, 2, 3	..
4	..	1, 2, 2, 3	..
5	104·7°+	..	..
9	106·0°+	0, 1, 1, 3	..
12	..	0, 0, 1, 2	Practically all eggs showing recognizable lesions.
15	105·0°+	0, 0, 1, 2	
19	..	0, 1, 2, 2	
23	104·1°+ (no sec. rise)	2, 2, 3, 3	..
26	..	0, 1, 2, 2	..
35	..	0, 1, 1, 2, 2, 2	Incubation at 36°–37° commenced.
43	..	1, 1, 3, 4	..
46	..	0, 1, 1, 1, 2	Focal lesions countable.
52	..	1, 2, 2, 3	Embryo dead between 3rd and 4th day.
53	..	1, 1, 2, 2	..
63	..	0, 0, 1, 1, 2	Embryo dead with hæmorrhagic encephalitis on 3rd day.
64	102·8°—	..	..
67	102·4°—	0, 2, 3, 3	..
70	..	1, 2, 2, 2	Muscle hæmorrhages first noted.
73	104·6°+, 103·4°—, 103·2°—, 102·7°—	..	Dead at 48 hours.

+ = presence of symptoms—nasal discharge, sneezing—etc., in ferrets inoculated. — = no symptoms observed.

Highest temperature reached during 5 days after inoculation shown in °F.

These changes in activity are due to changes in the virus, not in the susceptibility of the eggs. On one occasion eggs were inoculated on the same day with material from the 38th, 63rd, 69th and 74th passages with the following results :

38*th passage.*—Glycerinated material 16 weeks old gave typical discrete lesions with living embryos when the eggs were opened after 4 days.

63*rd passage.*—Glycerinated material 5 weeks old gave typical large foci with living embryo at 2 days. At 3 days one embryo was alive, but showed early brain hæmorrhages; another was dead with typical massive hæmorrhage in the brain.

69*th passage.*—Dried material from this passage about a fortnight old gave only a small number of foci, but these were large and typical. Two embryos were alive on the 2nd day ; two others were dead on the 3rd day, one showing gross brain hæmorrhage and slight muscle hæmorrhages, the other moderate lesions in the brain, but very extensive bleeding into the feather follicles of the skin.

74*th passage.*—The current passage from fresh egg material gave two eggs showing distinct early lesions with living embryos at 20 hours. The other two were both dead at 44 hours, with widespread hæmorrhages in skin and muscles. One showed moderate hæmorrhages into the brain, the other none.

Comparison with Table II will show that the preserved material has in each case retained the characteristic activity manifested at the passage concerned.

CHARACTERISTICS OF EGG STRAIN OF INFLUENZA VIRUS AFTER ABOUT SIXTY-FIVE PASSAGES.

The egg-passage strain of influenza virus is now something very different from the original strain and from the mouse-passage strain which has been maintained over the same period. Almost their only common feature is their antigenic identity. In the detailed description of the egg virus which follows its characteristics are those of the generations between the 63rd and 70th passages.

Macroscopic Appearances on the Developing Egg.

If the supernatant fluid from a typical membrane ground with 5 c.c. of broth and centrifuged is used as a stock virus suspension, a 1 : 1000 dilution of this will nearly always give a small number of discrete foci (5–100) when eggs are inoculated with 0·05 c.c. quantities. The lesions are well developed at 48 hours. At this period they appear as small opaque thickenings from about 0·2 to 0·8 mm. in diameter, usually distinctly protruding above the general surface of the membrane (Fig. 2). They are always surrounded by a haze of variable extent, which represents the accumulation of inflammatory cells in the mesodermal layer. Secondary " satellite " foci are quite common even as early as the second day, and frequently make counting of the number of primary foci very difficult. With very small numbers of primary foci it is usually easy to distinguish them from secondary satellites, but with large numbers it is practically impossible.

With higher concentrations of virus the foci become correspondingly crowded together, but one never obtains a completely confluent lesion. With undiluted stock virus parts of the membrane at least will always show discrete foci. A heavily infected membrane is always thickened and œdematous.

If infected eggs are opened on the 3rd day after inoculation the embryo will be found to be either obviously sick or dead. In either case hæmorrhagic lesions in the brain are evident. Dead embryos show a conspicuous purple bulging of the cranial region (Fig. 3), and occasionally one finds almost equally obvious changes in still living embryos. As a rule the earlier brain lesions take the form of small petechiæ scattered over the cerebral hemispheres or optic lobes. In one embryo both lateral ventricles were filled with blood, but the rest of the brain was practically unaffected. There are no significant differences between eggs incubated at 36°–37° C. and those incubated at 39·5° C.

Apart from the brain hæmorrhages there are no characteristic lesions. One or two dead embryos showed scattered small hæmorrhages in the skin and muscles, but these were absent in the majority during the period being considered. After the 71st passage they became the predominant type of lesion.

Infectivity for Animals.

The infectivity of the egg virus for mice has been extremely and rather surprisingly constant throughout, always causing definite mild lesions in a proportion at least of the 4 to 6 mice inoculated at each test. Only on very rare occasions have completely consolidated lungs been observed. The lesions produced by inoculation with these egg-membrane emulsions are specific, since they can be transmitted in series from mouse to mouse, and are normally neutralized by specific sera such as F22. Further, many inoculations with egg-membrane emulsions not containing influenza virus have never given the characteristic plum-coloured areas of consolidation.

The infectivity for ferrets on the other hand has become much lower, and in the later generations under consideration has almost been lost. The 64th and 67th generations produced no symptoms and maximum temperatures of 102·8° and 102·4° F. in the ferrets inoculated. Four ferrets were inoculated with material from the 73rd egg passage. One, F36, developed distinct mild symptoms and showed a definite but slightly atypical febrile reaction, the highest temperature being 104·6° F. Two others had no symptoms, but a small rise of temperature to 103·4° and 103·2° on the second day after inoculation. The fourth showed neither symptoms nor any temperature rise above 102·7° F.

In view of the great increase in pathogenicity for the egg it was of interest to test the egg-passage virus by intratracheal inoculation in fowls. Two young adult white Leghorn roosters were inoculated by this route with material from the 67th passage. Neither showed any symptoms referable to the respiratory tract, but one lost weight considerably, and a fortnight after inoculation provided a serum capable of inactivating completely undiluted egg virus suspension with which it was mixed in equal volume. The other bird remained in good condition and its serum showed no neutralizing power.

Distribution of Virus in the Embryo.

If a typical membrane is ground with 5 c.c. of broth and the supernatant fluid tested on eggs, 0·05 c.c. of a 1 : 1000 dilution will nearly always give a

small number (5–40) of discrete foci. Before the appearance of the hæmorrhagic brain lesions a few tests of liver and brain emulsions have shown no virus in these organs in embryos with typical 3-day membrane lesions.

Tests of the mixture of blood and softened brain removed from the cranial cavity of a dead embryo of the 63rd passage showed a good deal of virus to be present, sufficient to give 10 foci with a 1 : 100 dilution of about half the brain emulsified in 5 c.c. of broth, *i. e.* considerably less than was present in the membrane lesions. The virus is present in the brain before lesions are evident, but in smaller amount. An emulsion from the brain of an embryo removed 2 days after inoculation showed about 25 foci only with undiluted material.

Later work indicated that in all probability a considerable part of the virus present in the brain lesions was associated with the blood rather than with the nervous tissues. An egg inoculated with 73rd generation virus was opened at 20 hours after inoculation. A slit was made in the chorio-allantois and the main allantoic vein picked up with a loop of thread. By introducing a fine-pointed capillary pipette into the bulging distal portion, about 0·4 c.c. of blood was obtained and mixed with an approximately equal volume of 2 p.c. citrate in saline. A portion of this was tested as whole blood and a portion centrifuged, the supernatant fluid being tested on eggs. Table III indicates that only about 3 p.c. of the virus present remained in the supernatant fluid. There is a considerable amount present in or on the blood-cells at this stage, and presumably the amount increases by the time the embryo dies.

TABLE III.—*Presence and Distribution of Virus in Citrated Blood of Embryo at 20 hours.*

Material.	Dilution. 1 : 2.	1 : 200.
Whole blood . . .	300± .	2/3, 3/4
Supernatant fluid . . .	11/13, 6/6 .	0

See notes under Table IV.

Persistence of Antigenic Character on Egg Passage.

Very satisfactory neutralization tests can be carried out by the egg membrane technique, and a study of the influenza virus-antiserum reaction by this method will be described in a subsequent paper. Here it is only necessary to present evidence that the modified egg-passage virus is still antigenically identical with the original strain. Tests have been made with two ferret immune sera, one, F1, from a ferret inoculated with the original human material only, the other, F22, receiving mouse-passage virus.

For the egg tests suitable dilutions of virus, the supernatant fluid from an emulsion of egg membrane ground in 5 c.c. broth, and serum were made in saline, mixed in equal volumes, and left at room temperature for about 30 minutes before commencing the egg inoculations. The mixtures were inoculated in 0·05 c.c. quantities into 3 eggs each and the results read after 2 days' incubation at 36–37° C. The approximate counts of primary foci obtained are shown in Table IV. No detailed discussion of the immunological results

will be given here, but the findings make it clear that both sera are capable of inactivating large amounts of virus of either the mouse- or egg-passage strains. There has certainly been no gross change in the antigenic character of the virus during its varied passages since it was responsible for the initial human infection.

TABLE IV.—*Comparative Serum Neutralization Tests in Mice and Eggs.*

1. Ferret serum I convalescent after inoculation with original human material.

Mouse Test :

Mouse virus dilutions.	Serum dilutions. 1 : 2.	1 : 20.	1 : 200.	Saline control.
1 : 2	0, 0	0, 0	1, 2	3, 4, 4
1 : 20		..		3, 4
1 : 200		..		2, 2

Egg Test :

Egg virus dilutions.	Serum dilutions. 1 : 2.	1 : 20.	Saline control.
1 : 2	2/3, 4/13	+	..
1 : 20	..	13/28, 20/20	..
1 : 100	..	..	+
1 : 1000	..	..	24/32, 14/22

2. Ferret serum 22 inoculated twice with mouse passage material.

Mouse Test :

Mouse virus dilutions.	Serum dilutions. 1 : 20.	1 : 200.	1 : 2000.	Saline control.
1 : 2	0, 0, 0	0, 0, 2	3, 3, 4	3, 3, 4, 4
1 : 20	..	0, 0, 0	0, 0, 0	1, 2, 3, 3
1 : 200	..	..	..	1, 1, 2, 2

Egg Test :

Egg virus dilutions.	Serum dilutions. 1 : 200.	1 : 2000.	Saline control.
1 : 2	5/5, 0/1, 7/21	72/83, +, +	..
1 : 20	..	10/20, 2/2, 4/4	..
1 : 200	..	..	41/46, +, +
1 : 2000	..	..	4/4, 4/5, 1/2

Notes.—Results of mouse tests are expressed according to the convention adopted by Laidlaw, Smith, Andrewes and Dunkin, 4 representing complete consolidation of both lungs, 0 no consolidation, and 1, 2 and 3, intermediate degrees. Egg results are shown as the number of foci present on the egg membrane. Owing to the satellite phenomenon it is usually impossible to give a single figure, but it is not difficult to obtain counts representing (*a*) the smallest reasonable number of primary centres and (*b*) the highest reasonable count. These are shown in the form *a*/*b*. Where counting was impossible but lesions were present, + is used.

HISTOLOGY OF THE CHORIO-ALLANTOIC MEMBRANE LESIONS.

Sections have been prepared from 2- and 3-day membranes at various stages in the course of egg passage, and more detailed studies have been made of the active lesions occurring from the 60th–70th passages. As is to be expected from the macroscopic appearances, considerable changes in the histology of the lesions are recognizable with the development of more active pathogenicity for the egg. In certain respects the changes are analogous to the differences found in the histological structure of ectromelia lesions developing at different temperatures of incubation (Burnet and Lush, 1936).

In the earlier stages membranes showing exceptionally "good" lesions were naturally chosen for section, so that the description given will not be directly applicable to the average membrane at this period. However, the membranes used nearly always showed lesions of varying intensity, and one can be reasonably certain that the general structure of the relatively well-developed lesions and of the less definite average lesions is similar. The first change visible at 20–24 hours is the one common to all early virus lesions—a small region of proliferation in the ectodermal layer. The central cells of the group show degenerative changes involving both cytoplasm and nucleus, and an occasional leucocyte may be seen penetrating the area of commencing necrosis. The further development of the lesion follows the usual course of weak ectodermal lesions. The deeper layers of ectoderm proliferate beneath the initially infected cells, and eventually throw off a necrotic mass of dead ectodermal cells usually containing some leucocytes and red blood-cells. The difference between the well-developed and the weaker lesions depends only on the extent to which the proliferative-necrotic processes advance before being checked by the subjacent proliferation and cornification of uninfected ectodermal cells. In the larger lesions at 2 days the intermediate zone is composed of degenerating cells which are obviously infected, but by the next day there is practically always a well marked line of demarcation between the necrotic material and the cornifying ectoderm (Fig. 5). Inflammatory changes are slight throughout the process.

The lesions produced by the virus of the later passages commence in similar fashion, but it is soon evident that the virus has a more damaging effect on the ectodermal cells. The proliferation is more irregular and the damaged cells are early invaded by leucocytes so that the integrity of the ectodermal layer is lost. As the lesion develops we find several processes at work which combine in varying degrees to produce the final lesion. These are (*a*) the usual proliferation followed by degeneration and necrosis of the ectodermal cells, (*b*) an intense leucocytic migration toward and amongst the infected ectodermal cells, and (*c*) proliferation and possibly infection by the virus of cells derived from the endothelium of the superficial capillaries. The two chief types of lesion resulting are illustrated in Figs. 4 and 6, and diagrammatically in Fig. 1. In the first the intense accumulation of mesodermal inflammatory cells results in a rupture of the infected ectodermal layer, and a pouring out of semi-necrotic material through a relatively narrow opening. The second type does not show the localized rupture, but there is a general infiltration of leucocytes through the whole of the proliferating ectoderm with necrosis of

the outermost layers. Since death of the embryo always occurs under these conditions before the 3rd day there is no stage of repair to be described.

Careful search has been made for cells showing inclusion bodies of any of the recognized types associated with virus lesions. The only appearances at all suggestive were distinctly rare nuclear changes in ectodermal cells, some of which approached very closely the typical form of an intranuclear inclusion. Nuclear degeneration is usual in the cells near the necrotic cap of a 2-day focus, and is often shown by enlargement of the nucleolus, which with Mann's stain develops a reddish central mass with distinct dark purple thickenings

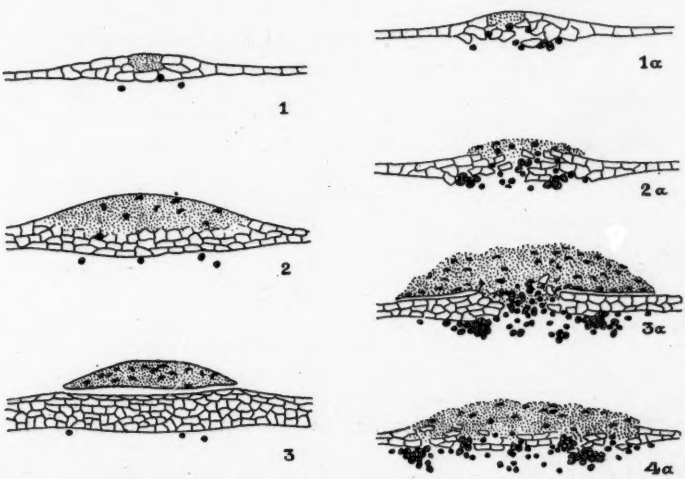


FIG. 1.—Diagrams illustrating the development of the ectodermal lesions on the chorio-allantois. 1, 2 and 3 represent successive stages in the development of a focus by virus of about 30–40 passages; 1*a*, 2*a*, 3*a* and 4*a* show similar stages with more active virus (65–70 passages), two alternative final stages, 3*a* and 4*a*, being shown. × 150. Ectodermal cells are shown white, necrotic material stippled, eosinophil leucocytes by black dots, circular if normal, distorted when necrotic, and mononuclear cells apparently derived from capillary endothelium by small shaded circles.

at the edge. This appearance is relatively common, and it appears likely that the much rarer appearances simulating true intranuclear inclusions represent a final stage of nucleolar enlargement, with partial disintegration and migration of the basically staining edge of the enlarged nucleolus to the nuclear membrane. The appearances of the presumed early stages are quite different from those of the true intranuclear inclusions produced by laryngotracheitis virus, in which the inclusion can be seen to develop alongside the nucleolus. The fully developed form found in influenza virus lesions has, however, an appearance which would certainly pass for a typical inclusion if seen in a laryngo-tracheitis lesion. The chromatin is accumulated in irregular thickenings of the nuclear membrane, and the centre of the nucleus is filled with an amorphous, slightly acidophilic mass with a soft edge not quite reaching the nuclear membrane.

Such cells were so rare that one can hardly assume that the production of nuclear inclusions is a characteristic of the virus.

DISCUSSION.

Although the virus of epidemic influenza has only been under study for a year or two, there is already considerable evidence of the lability of its virulence. Mice, for instance, can only be infected after the virus has undergone a few passages through ferrets. Normally pneumonia is not produced in ferrets, but by suitable lung to nose passage, strains which regularly induce pneumonia can be developed. If Laidlaw's (1935) hypothesis in regard to the relation between swine influenza and the pandemic of 1918–1919 is correct, the adaptation of the human strain to swine represents a striking natural manifestation of this lability.

The results of egg passage provide an almost equally striking example. If we compare the final egg strain with a mouse-passage strain there seems to be very little in common beyond an identical antigenic structure. The egg strain is highly virulent for chick-embryo tissues, producing discrete lesions on the egg membrane in number proportional to the concentration of virus, and killing the embryo within 3 days with intense vascular lesions, particularly in the brain. The virus appears in the embryo blood within 20 hours of inoculation and is apparently mainly present in or on the blood-cells. It has presumably developed a capacity to infect and multiply in the cells of the capillary endothelium as well as in the ectoderm of the chorio-allantois.

The egg-passage virus was derived from a ferret strain and has never been passaged through mice, but has retained its initial virulence for the mouse, although its pathogenic action on ferrets has greatly diminished and is now almost negligible.

The corresponding mouse-passage strain is fully virulent for mice, and produces typical symptoms and temperature reaction in ferrets. When inoculated on the egg only the slightest evidence of proliferative changes is produced, although the virus persists and can be carried on by successive passage. No pathogenic effect on the embryo proper can be detected. The mouse-passage strain thus behaves on the egg precisely as the original ferret virus which was used to start the main passage series, and like the strain used

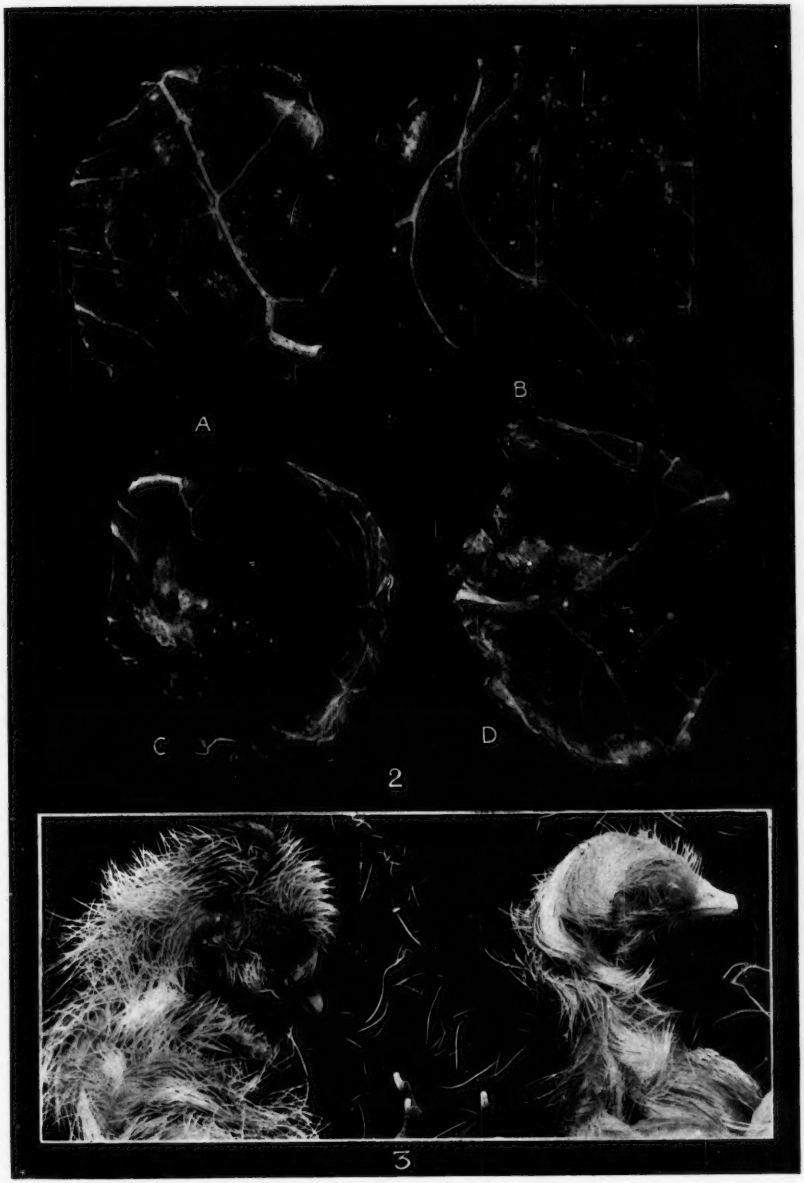
FIG. 2.—Membranes obtained in a titration of 59th passage egg virus. A and B received 1 : 100 dilution of stock emulsion, C and D, 1 : 1000 dilution. A, B and C show typical discrete foci. D shows several patches of secondary foci without distinct primary lesions.

FIG. 3.—Embryo dead on 3rd day after inoculation with 62nd passage egg virus, showing characteristic appearance of hæmorrhagic brain lesions. For comparison an embryo of similar age from an egg inoculated 4 days previously with psittacosis virus is shown on the right.

FIG. 4.—Egg membrane 4730, 63rd generation, 2 days after inoculation. Showing a typical satellite system with central primary lesion and smaller outliers. × 65.

FIG. 5.—Egg membrane 2593, 3rd generation, 3-day lesion. Showing ectodermal proliferation with only moderate inflammatory response and necrosis limited to a cap of superficial cells. × 250.

FIG. 6.—Egg membrane 4760, 64th generation, 2-day lesion. Showing intense inflammatory response and pouring out of necrotic and inflammatory cells to form superficial cap over the lesion. This section served as a model for Diagram 3*a* in Fig. 1. × 250.



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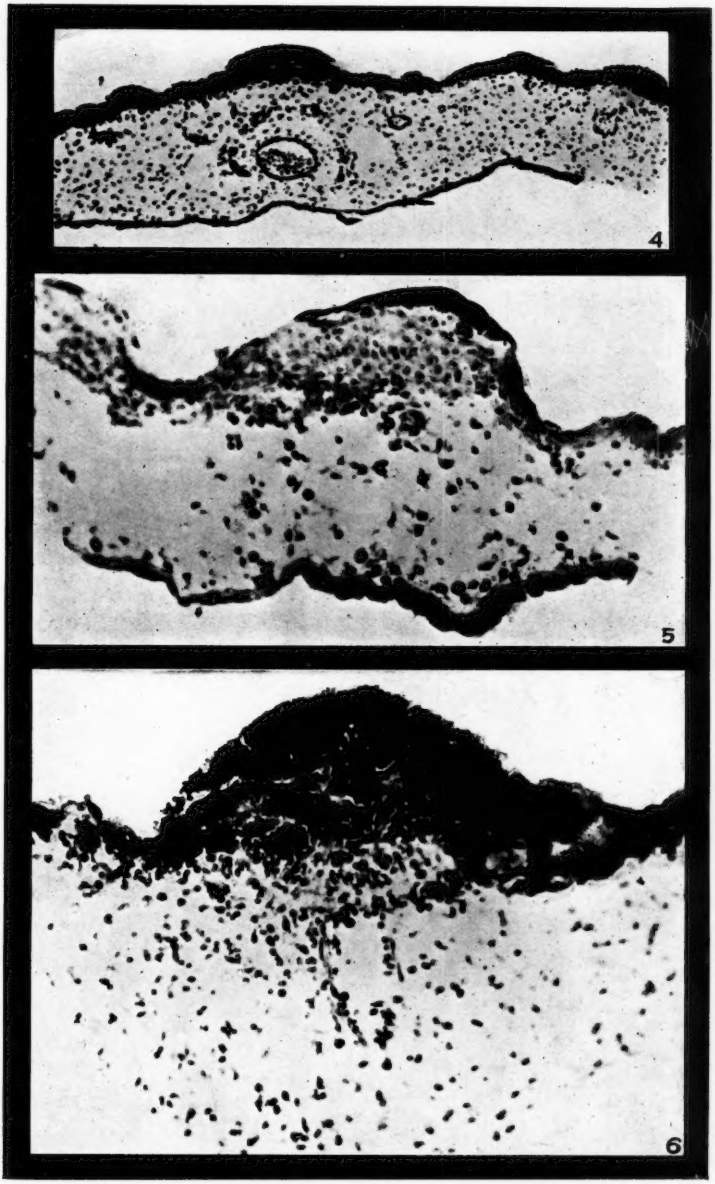

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by Wilson Smith in his work on the same subject. If our present experience is a just guide, it requires 30 or 40 consecutive egg passages before the virus produces satisfactory lesions on the membrane, but after that there is a steady increase in the pathogenicity for the egg. Up to the time of writing there has been no indication that the limit of activity has been reached. With the current passage strain death of the embryo takes place so early that the focal membrane lesions have little time to develop, and are much smaller than those obtained about the 60th passage.

It seems likely that the development of a strain of influenza virus highly pathogenic for the developing egg will provide an experimental method of some value for academic investigations, and perhaps as a source of virus for any type of human vaccination which may be developed in the future.

SUMMARY.

1. Virus from a typical case of epidemic influenza has been propagated for more than 70 generations in developing eggs.

2. The lesions produced in the initial passages were trivial, but the pathogenicity of the virus for the embryo has steadily increased. By the 73rd passage all embryos were killed within 48 hours with multiple hæmorrhagic lesions in many tissues.

3. Well-defined focal lesions, whose number can be used as an index of the amount of virus inoculated, are produced on the chorio-allantoic membrane by the later generations.

4. The histology of the chorio-allantoic membrane lesions is described and illustrated.

5. The virus has retained its original immunological characteristics.

REFERENCES.

ANDREWES, C. H., LAIDLAW, P. P., AND SMITH, W.—(1935) *Brit. J. exp. Path.*, **16**, 566.

BURNET, F. M.—(1933) *J. Path. Bact.*, **37**, 107.—(1935*a*) *Med. J. Australia*, **2**, 651.—(1935*b*) *Ibid.*, **2**, 687.—(1936) *J. exp. Med.* (in the press).

Idem AND LUSH, DORA.—(1936) *J. Path. Bact.* (in the press).

FRANCIS, T.—(1935) *Proc. Soc. exp. Biol., N.Y.*, **32**, 1172.

LAIDLAW, P. P.—(1935) *Lancet*, **1**, 1118.

Idem, SMITH, W., ANDREWES, C. H., AND DUNKIN, G. W.—(1935) *Brit. J. exp. Path.*, **16**, 275.

SMITH, W.—(1935) *Ibid.*, **16**, 508.

Idem, ANDREWES, C. H., AND LAIDLAW, P. P.—(1933) *Lancet*, **2**, 66.

OBSERVATIONS ON THE EFFECT OF LOUPING ILL VIRUS ON THE DEVELOPING EGG.

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In 1933 a small number of experiments carried out in collaboration with Mr. I. A. Galloway at the National Institute for Medical Research showed that louping ill virus could be propagated in the developing hen's egg. As part of a systematic study of virus lesions in this situation a good deal of further work has been done on louping ill virus in Melbourne during the last two years, the results of which are reported in this paper.

The technique used for egg inoculation has been frequently described in earlier papers, and no modifications were introduced for the present work. The virus used was a strain originally obtained from Moredun by Mr. Galloway, and since maintained by intracerebral passage in mice. It has consistently produced fatal infection in mice with characteristic symptoms and is regularly inactivated by known immune monkey sera, two lots of which were available through the courtesy of Mr. Galloway and of Dr. T. M. Rivers respectively. In nearly all experiments inoculations were made with membrane filtrates prepared from fresh or glycerinated mouse brains by Elford's technique, the final filtration being through a gradocol membrane of approximate average pore diameter of 0·7μ.

Preliminary work showed that the virus could readily be propagated from egg to egg, subinoculations being made either from chorio-allantoic membrane emulsions or from the embryo brain or liver. Though it was obvious that certain pathological changes were occurring both in the chorio-allantois and in the embryo, their nature was not ascertained until a more detailed study on eggs inoculated at various stages of incubation had been carried out.

CHORIO-ALLANTOIC MEMBRANE LESIONS PRODUCED BY LOUPING ILL VIRUS.

The macroscopic appearances of the lesions vary very strikingly according to the age of the embryo when the inoculation is made. The following results were obtained when a 1 : 100 dilution of a mouse brain filtrate was inoculated into eggs of different ages. All the eggs were opened three days after inoculation.

Ten-day eggs showed no specific focal lesions. There was considerable œdema, which was more or less restricted by irregular bands of cellular opacity, giving a quilted appearance. These opacities frequently radiated from a non-specific lesion beneath the point of inoculation.

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Inoculated at 11 days one egg showed a lesion precisely similar to the average 10-day lesion. The other showed more definite changes. There was uniform cellular and œdematous thickening, with ectodermal lesions in the form of small rather diffuse and confluent, but distinctly focal opacities.

At 12 days the focal lesions showed to the best advantage. There was only a moderate œdema, and the specific foci stood out well as small (0·2–0·3 mm.) white spots thickly scattered over the membrane. Some of the larger spots near the periphery appeared to be surrounded with satellite foci, and it was hard to be certain whether the closely set foci over most of the membrane all represented initial infections, or resulted from diffusion of virus particles liberated from the true primary foci.

Lesions from the eggs inoculated at 13 days showed a general resemblance to 12-day lesions, but were smaller and less distinct. Inoculations made on the 14th day had very little effect, the membrane showing only a vague powdering with minute focal opacities and very little œdema.

In all recent work in which chorio-allantoic lesions have been studied, only eggs at the 12th day of inoculation and showing by transillumination a normal degree of development have been used. In such eggs, when virus dilutions approaching the limit of activity are employed, well-defined discrete foci may occasionally be observed. Much more frequently there is a more or less diffuse formation of secondary foci, which in the highest dilutions can almost always be seen to represent a small number of centres of spread. Sometimes a distinctly larger primary focus may be seen surrounded by regularly distributed satellite foci, but as often there is a roughly circular patch of close-set tiny foci with no distinguishable central point. If three or four eggs are used for each 10-fold dilution of virus, there is usually no difficulty in making an approximate count of the number of primary foci or satellite centres in the highest active dilution. As will be shown in a later section, the number of such foci is probably approximately equivalent to the number of active virus particles present.

HISTOLOGY OF LOUPING ILL LESIONS IN THE CHORIO-ALLANTOIC MEMBRANE.

The chief histological interest attaches to the focal lesions produced most clearly in 12-day eggs. The tiny focal opacities which are characteristic of louping-ill infection can just be seen with a lens when the egg is opened 20–24 hours after inoculation. Histologically the essential features of the lesion are better seen at this period than in later sections. Each focus is represented in the section by a spindle-shaped thickening of the ectodermal layer. The thickening is due mainly to proliferation, giving in the centre of the spindle a layer 3 cells thick. When the section passes through the centre of the lentiform ectodermal thickening which constitutes the specific focal lesion, a small region of necrosis is found at the centre of the lesion. In two instances a little ragged hiatus in the surface indicated where what was presumably the cell initially infected by the virus particle had completely disintegrated. The necrosis spreads outward, and at 20 hours appears to involve probably a quarter of the cells in the lesion. It is evidenced (*a*) by the appearance of swollen cells with coarsely granular or almost vesicular protoplasm, (*b*) by nuclear changes of

various sorts—general loss of structure and staining power, extrusion of nucleolus. Outside the zone of necrosis the cells appear normal, but are obviously proliferating rapidly, and where mitoses are present a considerable proportion appear to be abnormal, showing a reduced number of short thick-set chromosomes. In sections stained by Mann's method red-staining bodies are fairly regularly present in the cytoplasm of cells undergoing necrosis. They are of variable size and appearance, and although some have a close resemblance to the bodies described by Hurst (1931) in mouse brain lesions, it seems unlikely that they have any specific significance.

On the second day one finds the usual slight general proliferation and cornification, with well-marked specific focal proliferations. Many of these roughly spindle-shaped ectodermal proliferations appear to be composed of healthy cells with a little debris infiltrated by leucocytes on the free surface. A smaller number show in the centre of the proliferating cells an area of necrosis with liquefaction of the cells sometimes sufficient to give rise to a distinct cyst. In almost all instances the necrotic area is not as in the first-day lesions on the free surface, but is separated from it by a layer or two of apparently normal cells. One gains the impression that almost all the cells forming the earliest foci have been destroyed under the combined influence of virus necrosis and leucocytic infiltration, and that cells of the existing lesions are mostly the descendants of the uninfected deep layer cells, which are seen beneath some of the primary lesions on the first day. The cells of this secondary lesion are relatively resistant to infection by louping ill virus, just as similar cells are to psittacosis and ectromelia virus infections.

By the third day there is little evidence of any actively necrotic process. The foci appear as ectodermal thickenings with a cornified surface, on which some of the necrotic debris of the initial lesion may remain.

Throughout there is, in addition to the ectodermal proliferative and necrotic lesions, a considerable amount of inflammatory mesodermal change which follows the usual sequence—œdema and migration of bilobed eosinophil leucocytes in the earlier stages, with a predominantly fibroblastic reaction later.

VISCERAL LESIONS IN THE INFECTED EMBRYO.

If eggs inoculated with an amount of virus sufficient to give a confluent or almost confluent membrane lesion are left to incubate, nearly all die within a week, usually on the sixth day. The dead embryos show characteristic lesions, which are also and more satisfactorily observed in eggs opened on the 5th or 6th day after inoculation, *i. e.* just before the embryo dies. The following appearances are frequently observed, but usually only a certain number of them are to be found in any one embryo: The blood-vessels of the chorio-allantois may be smaller than normal, with a sluggish circulation, probably indicating diminished blood-volume; or more rarely they may be distended with blood of a peculiar opaque salmon tint of low hæmoglobin content and containing mostly immature red cells. Every degree of jaundice may be encountered involving both the embryo and the other contents of the egg. If jaundice is severe the embryo invariably appears smaller than normal, emaciated and anæmic-looking. With lesser grades of jaundice and sometimes in its

absence there may be very gross œdema of the embryo, particularly the body-wall. On opening the body-cavity of the embryo changes in the liver are almost always visible. Either it is a deep greenish-brown colour without necrotic areas, of if normally tinted shows extensive white or yellow necrotic patches often involving the whole of the organ. As a rule the dark green livers are associated with jaundice of the embryo and the appearance of excessive bile-pigment in the intestinal lumen; the necrotic livers are more characteristically associated with œdema of the body-wall of the embryo. There are, however, frequent exceptions, and intermediate conditions to be observed.

Table I summarizes the data from six eggs chosen to show most of the different combinations of lesions which have been encountered. Type 4 is common in eggs inoculated on the 10th day, but very rare with eggs inoculated on the 12th day. The latter more commonly show types 1, 2 or 6.

TABLE I.—*Visceral Lesions of Embryos in Eggs Inoculated with Louping Ill Virus.*

No.	Days after inoculation.	Membrane lesion.	Liver.		Excess bile-pigment in intestine.	Embryo.		
			Colour.	Necrosis.		Jaundice.	Œdema.	Emaciation.
1	6	++	Dark	+	+	+	—	—
2	6	++	Dark	—	+	+	—	—
3	6	++	Normal	+++	+	+	—	+
4	5	++	Normal	+++	—	—	+	—
5	5	++	Sl. greenish	++	+	±	—	+
6	5	++	Dark	++	+	—	—	±

Under membrane lesion ++ indicates semi-confluent lesion; degrees of liver necrosis are indicated by +++, practically complete; ++, more than a third of the liver necrotic; +, minor necrotic patches. In other columns + = present, — = absent, ± = slight or doubtful.

When an extensive lesion is produced on the chorio-allantois with such viruses as those of vaccinia, fowlpox, ectromelia or laryngo-tracheitis, death usually occurs in 4 to 6 days, and in the later stages necrotic patches on the liver are usual and there is some evidence of anæmia, but jaundice is never observed, and the liver changes are usually much less striking than with louping ill infections.

Virus is regularly present in the embryo blood and in the washed blood-cells at periods from the 2nd to the 6th days after inoculation. The usual method used for obtaining blood was to make a slit through the chorio-allantois, avoiding major blood-vessels, pick up the main allantoic vein and pass a sterile cotton thread around it. On drawing the vessel up with this thread the distal portion ballooned up sufficiently to allow a fine-pointed capillary pipette to be inserted and up to 0·5 c.c. of blood to be withdrawn. Another method used when it was desired to avoid any possibility of picking up virus from the chorio-allantois was to make a fresh opening in the shell away from the inoculated area, and to collect the blood oozing after reflection of the shell membrane in a Pasteur pipette. Both methods gave similar results, which are included in Table II.

There is not a great deal of virus present, but always sufficient to kill mice

TABLE II.—*Virus Content of Washed Blood-cells from 8 Infected Chick Embryos.*

Day after inoculation on which blood was taken.	Fate of mice inoculated with washed cells in concentration shown.
2nd .	5 p.c. 6, 7.
3rd .	1 p.c. 6, 7 ; 7, 7 ; 7, 7 ; 7, 7 ; 7, S.
5th .	1 p.c. 7, 7 ; 7, 7. 0·1 p.c. 9, S. : S, S.

Numbers in this and similar tables refer to number of days elapsing before death of mouse with typical symptoms of louping ill infection. S. = Survived.

inoculated intracerebrally with a 1 p.c. emulsion. A significant amount of virus can be liberated from washed blood-cells by lysing them in distilled water and testing the well-spun supernatant fluid. In this experiment about 0·4 c.c. of blood was obtained from the allantoic vein of an embryo 6 days after inoculation and showing well-marked visceral lesions. The blood-cells were washed twice with 10 c.c. of saline, and the deposit added in equal amounts (0·1 c.c.) to 10 c.c. of distilled water and saline respectively. Titrations on the egg membrane were made with (*a*) supernatant fluid after centrifuging the hæmolysed cells in distilled water ; (*b*) the cell emulsion in saline ; (*c*) the supernatant fluid obtained after centrifuging (*b*).

Table III shows the results obtained.

TABLE III.—*Virus Content of Blood-cells from Infected Embryo ; Effect of Hæmolysis with Distilled Water.*

Material.	Virus content (egg membrane test).	
	Undiluted.	Diluted 1 : 100.
(*a*) Supernatant from hæmolysed cells .	++ .	7, 6
(*b*) Cells in saline	++, ++ .	6, 3
(*c*) Supernatant from (*b*)	0, 0 .	..

Egg membrane lesions are indicated by a figure representing the number of primary foci visible on the membrane; ++, numerous primary foci ; +, positive membrane, but no estimate of number possible usually on account of diffuse secondary spread.

A number of blood examinations were made from embryos showing typical lesions. Hæmoglobin was estimated with a Hellige " Neoplan " hæmometer, the diluted material being spun for a minute or two to deposit nuclei, etc., before the supernatant was compared with the standard. Blood from the normal embryos of the same age gave readings of 4·6–5·4 g. hæmoglobin per 100 c.c. Most of the infected embryos gave figures from 3·0–4·0. Red cell counts did not appear to be unusually low, counts of 1·8, 2·2 and 2·8 millions from infected eggs comparing with 2·7 and 2·8 millions from normal embryos. Smears showed increased numbers of immature cells in nearly all the infected embryos, some with about 25 p.c. of cells with pale or slightly basophilic cytoplasm and larger nucleus than normal, as compared with the 1–2 p.c. of such cells in normal embryo blood. The blood of one embryo appeared a salmon colour in the vessels, and though it contained 2·8 million cells per c.mm. the hæmoglobin was too low to be estimated. The smear showed the majority of the cells to be achromic and a large number of very early forms were present.

No specific changes which could be ascribed to the virus were observed in any of the smears examined.

The constant presence of the virus in association with the blood-cells is very suggestive that one important part of the syndrome which follows infection of the embryo with louping ill virus is a hæmolytic anæmia. The necrotic liver lesions may be secondary to the blood changes, but more probably they are another manifestation of the direct attack of the virus. In any case the main effects of the virus on the embryo are on tissues other than those of the nervous system. Louping ill is usually regarded as a fairly strictly neurotropic virus. In the egg it is essentially viscerotropic.

COMPARATIVE TITRATIONS OF LOUPING ILL VIRUS IN MICE AND ON THE DEVELOPING EGG.

In Table IV are given the results of titrations of louping ill virus preparations done in parallel (*a*) by chorio-allantoic membrane inoculation and (*b*) by intracerebral inoculation in mice. Tenfold dilutions were made in ice-cold saline containing 0·024 p.c. of $CaCl_2$ (Ca-saline) and 0·05 c.c. amounts inoculated into 12-day eggs, and 0·03 c.c. amounts intracerebrally in mice. The egg lesions are denoted + when specific lesions were present, but not countable; figures are given when the number of primary foci and/or distinct satellite systems could be counted. The results of mice inoculations are shown by the number of days after inoculation before the mouse died or was killed with typical symptoms; S signifies survival for 14 days.

TABLE IV.—*Comparative Titration of Louping Ill Virus in Mice and Men.*

Preparation.	Test organism.	Dilution of virus. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	Calculated titre.
Mouse brain .	Mice .	7, 7, 7, 8 .	7, 8, 9, S .	S, S, S, S .	.. .	.. .	$2{\cdot}5\times10^{4}$
filtrate (1)	Eggs .	.. .	50+, + .	+, 18 .	.. .	.. .	$3{\cdot}6\times10^{6}$
Ditto (2) .	Mice .	6, 6 .	7, 7 .	7, 7 .	8, S .	.. .	$1{\cdot}6\times10^{5}$
	Eggs .	.. .	+, + .	+, 34 .	.. .	.. .	7×10^{6}
„ (3) .	Mice .	.. .	7, 7, 8, 8 .	7, 7, 8, 8 .	S, S, S .	S, S, S .	3×10^{6}
	Eggs .	.. .	++, ++ .	7+26+63 .	10, 7, 5* .	.. .	$1{\cdot}2\times10^{7}$
Egg .	Mice .	.. .	6, 7, 7 .	10, S, S .	S, S .	S, S .	$1{\cdot}1\times10^{5}$
membrane emulsion	Eggs .	.. .	+ .	20, 9, 6 + .	2,* 0 .	0, 0 .	4×10^{6}

* Membranes subinoculated to mice with positive result.

These results are typical of all the experiments in which virus preparations have been tested by both methods, and indicate that the egg-membrane is significantly more sensitive to infection by louping ill virus than the mouse brain.

SERUM-NEUTRALIZATION TESTS ON THE EGG MEMBRANE.

In view of the importance of the route of administration on the result obtained in serum-neutralization tests with certain viruses (see Sabin, 1934–5), it was of interest to see whether tests with louping ill virus on the egg membrane would give similar results to those obtained by the classical method

of intracerebral inoculation. Tables V and VI give comparative results with two sera, one a pooled serum from a number of rats inoculated intranasally with louping ill virus (Burnet, 1936), the other a highly active serum from an immunized monkey which was received through the courtesy of Dr. T. M. Rivers. In both cases the results show that the serum appears to be rather more active in the egg tests. With the rat serum the mouse tests indicate the neutralization of approximately 100 m.i.d's., while the egg results would indicate 2000. The monkey-immune serum similarly neutralizes around 10,000 m.i.d. by mouse test, 100,000 by egg test. However, if we recognize from Table IV that the minimal infecting dose for the mouse is something over 10 times that necessary to produce a single focus on the egg membrane, some such difference is to be expected. A point of considerable interest concerns the two eggs noted in Table VI. These showed no trace of membrane lesions, but one opened on the 5th day showed mild liver necrosis. Blood from each embryo was tested by intracerebral inoculation in mice, and that from the one with the liver lesions proved to contain virus. This suggests that some degree of dissociation *in vivo* can occur.

TABLE V.—*Comparative Test of Antibody Content of Pooled Immune Rat Serum by Both Methods.*

Serum.	Virus dilutions. 2.	20.	200.	2000.	20,000.	
Normal serum	..	5, 5, 6, 6	7, 7, 7, 7	8, 8, S, S,	S, S, S, S	Mouse test.
Immune serum	7, 7	10, S	S, S	S, S	..	
Normal serum	..	..	++	+, +	30, 12	Egg test.
Immune serum	..	..	0, 1	0, 0	..	

TABLE VI.—*Comparison of Antibody Content of Immune Monkey Serum in Mice and Eggs.*

Serum.	Virus dilutions. 2.	10.	50.	2000.	20,000.	200,000.	
Normal serum	..	..	..	7, 7, 8, 8	7, 7, 8, 8	S, S, S, S	Mouse test.
Immune monkey serum	7, 8	S, S	..	..	..	..	
Normal serum	..	..	..	++	7+, 26+, 63	7, 5, 10	Egg test.
Immune monkey serum	2, 7	0, 0	0,* 0†	..	..	..	

* Normal embryo, no virus in blood.
† Liver necrosis, virus present in blood.

DISCUSSION.

Though louping ill is one of the less satisfactory viruses to work with on the egg-membrane, the technique probably provides a more sensitive and accurate indication of the presence of the virus than intracerebral inoculation in mice, provided one has sufficient experience to distinguish the rather weak focal lesions from non-specific lesions and to separate primary from secondary foci. These difficulties are considerable, and most workers will undoubtedly prefer to retain the mouse for general work on louping ill virus. For the

detection of minute amounts of virus primary inoculation on to the egg-membrane followed after 5 days by subinoculation of an emulsion of the membrane intracerebrally in mice would probably be the most satisfactory method and might find an application in certain problems.

Hurst (1936) has recently suggested that the viruses of louping ill, yellow fever and equinine encephalomyelitis may be regarded as a fairly well-defined group (pantropic viruses, Group 2). They are distinguished from other neurotropic viruses by an ability to multiply in the blood or in some tissue intimately related to the blood, perhaps the vascular endothelium, and by their inability to enter the central nervous system along peripheral nerves other than the olfactory nerves. Another characteristic feature is the frequency with which infection of the nervous system may not appear till after the rest of the body is protected by circulating antibodies. It appears that we must regard these viruses as primarily viscerotropic, and by preference multiplying either in the circulating blood-cells or in the vascular endothelium and liver-cells. Attack on the nervous system at least in their natural hosts is a secondary and more or less accidental phenomenon. It will be seen that the behaviour of louping ill virus in the egg accords well with Hurst's interpretation. In at least three respects the egg lesions differ sharply from those produced by louping ill virus in experimental or natural hosts: (1) there is a primary proliferative-necrotic lesion of ectodermal epithelium, (2) the virus persists in the circulating blood, and (3) liver necrosis is a frequent feature. The first characteristic brings louping ill virus closer to that of equine encephalomyelitis, which can also produce ectodermal lesions in the guinea-pig foot, while the two latter ones suggest a relationship to the viscerotropic activities of yellow fever virus.

SUMMARY.

Louping ill virus may be propagated in the developing hen's egg, producing both local chorio-allantoic membrane lesions and general effects upon the embryo.

The number of focal lesions can be used as a sensitive but rather inaccurate indicator of the presence and amount of virus.

Serum neutralization tests can be carried out with the egg membrane as the indicator organism, the results agreeing fairly closely with those obtained by the method of intracerebral inoculation in mice.

Virus is constantly present in the circulating blood, is in part at least attached to the blood-cells, and is liberated from this attachment by the action of distilled water.

The lesions observed in the embryo are such as might be accounted for if the primary effect of this virus is to produce a hæmolytic anæmia with or without primary damage to liver-cells.

The almost exclusive viscerotropism of louping ill virus in this situation is discussed.

REFERENCES.

BURNET, F. M.—(1936) *J. Path. Bact.*, **42**, 213.
HURST, E. W.—(1931) *J. comp. Path.*, **44**, 231.—(1936) *J. Path. Bact.*, **42**, 271.
SABIN, A. B.—(1935) *Brit. J. exp. Path.*, **16**, 169.

THE IMMUNOLOGICAL RELATIONSHIP BETWEEN KIKUTH'S CANARY VIRUS AND FOWL-POX.*

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IN the course of work on the chemotherapy of bird malaria Kikuth and Gollub (1932) found that one of their passage strains of malaria had become contaminated by an agent highly fatal for canaries. They showed that a filterable virus was responsible which produced, on intramuscular inoculation in the canary, an inflammatory necrotic local lesion and a generalized infection through the blood. This virus has since then been used fairly extensively as a suitable object for the study of general virus problems. With the exception of psittacosis it is the largest virus so far known (Burnet [with Barnard and Elford], 1933). It is a very convenient agent for demonstration by dark-ground observation or ultraviolet microphotography, or by staining methods such as the Viktoria-Blau method developed by Herzberg (1934). The last-named author used this method to show directly the multiplication of the virus particles in the mononuclear cells which invade the necrotic areas in the infected canary.

Burnet (1933) concluded that Kikuth's virus was a member of the bird-pox group, basing this conclusion on the following grounds: The skin over inoculated areas showed thickening of the epithelium, with most of the cells containing cytoplasmic inclusions closely resembling the Bollinger bodies of fowl-pox. The virus particles were large, about 0·17μ in diameter, and like fowl-pox, the virus retained its activity well on keeping. Lesions were produced on the chorio-allantoic membrane of the developing egg showing a general resemblance to those of fowl-pox. Finally one strain of fowl-pox amongst several tested produced on intramuscular inoculation into the canary an infection with a longer incubation period than, but otherwise pathologically similar to, that produced by Kikuth's virus.

Later, in 1933, Burnet (unpublished) carried out a few experiments with material from spontaneous outbreaks of bird-pox in wild sparrows. Crusts from the lesions on these birds were obtained through the kindness of Dr. C. A. McGaughey, and no difficulty was experienced in transmitting the infection to canaries by scarification, or in subsequent passages by intramuscular inoculation of the exudate from the subcutaneous tissue. The virus was less virulent than Kikuth's, but produced almost identical pathological changes. The subcutaneous tissue fluid, which was a very satisfactory material for the preparation of membrane filtrates of Kikuth's virus, was almost useless for

* This work was carried out under a grant for work on virus diseases from the Commonwealth Government Department of Health and the Rockefeller Foundation.

this purpose with the sparrow-pox strain. Examination of this tissue fluid by Mr. J. E. Barnard showed that though the virus particles were of similar size they tended to be clumped together, unlike the discrete particles of Kikuth's virus. This observation accounted for the difficulty of filtration, and incidentally established another point of resemblance to fowl-pox virus, which is also difficult to filter through gradocol membranes. Gaede (1935) has also expressed the same opinion that Kikuth's virus is a bird-pox strain. He found that a typical strain of canary-pox, when transferred from canary to canary by intramuscular injection of infected blood, entirely reproduced the picture described by Kikuth.

Immunological work with the bird-pox viruses is rendered difficult by the limitations of host specificity, and the almost complete absence of demonstrable antibodies in the sera of immune or hyperimmune birds. Inoculation of canary virus is invariably fatal to canaries and produces no lesions in fowls, so that it was impossible to compare the antigenic structure of the virus with that of fowl-pox by orthodox methods.

The development in this laboratory of the technique of quantitative virus titration on the chorio-allantoic membrane suggested the possibility of making a direct comparison of the two viruses on the same indicator organism, the developing egg. It will be shown that by this method virus-inactivating antibodies can be demonstrated in fowl-pox hyperimmune sera and in the serum of a rabbit inoculated with large amounts of canary virus. Both these sera were active against both viruses; in each case the homologous virus was inactivated to a slightly greater degree than the heterologous one. This demonstration of close immunological relationship appears to provide final proof that Kikuth's canary virus is a typical example of the bird-pox viruses.

EXPERIMENTAL DETAILS.

Viruses.

The stock virus strains used were (*a*) Kikuth and Gollub's original virus obtained from Dr. Kikuth in 1932, and maintained by egg membrane passage since 1934. The strain is still fully virulent for canaries, producing typical lesions. (*b*) A fowl-pox strain obtained from Dr. H. E. Albiston, originally isolated from a turkey, but fully virulent for fowls, producing typical local and generalized lesions. Considerable difficulty was experienced in initiating egg passage with this strain owing to difficulty in obtaining active filtrates. Eventually an active preparation was made by treating the supernatant fluid with 0·5 p.c. phenol and a trace of gentian violet, and since then the strain has been maintained by egg-membrane passage. (*c*) The vaccine virus used was the egg-passage strain of Levaditi's neurovaccine used recently by Dr. E. V. Keogh for immunological work (Keogh, 1936) and kindly supplied by him.

Antisera.

Fowl-pox antisera were prepared by hyperimmunization of adult fowls. The serum used in all the experiments cited (H23) was obtained from a hen which was inoculated on the comb by scarification and intramuscularly with 2 c.c. of an emulsion of egg membrane ground with 5 c.c. broth. Typical lesions developed on the comb, and three weeks after the initial inoculation a course of three more intramuscular injections of fresh egg membrane material was commenced. Ten days after the last inoculation the fowl was bled from the carotid artery. Control fowl serum was obtained from birds whose susceptibility to infection was subsequently tested and found normal.

A rabbit was immunized against canary virus by inoculation with material prepared from

well-developed egg membrane lesions. Five freshly removed membranes were ground finely with about 10 c.c. broth and spun in an angle centrifuge for 10 minutes. The supernatant fluid was then removed to flat 4-mm. tubes and centrifuged in the angle centrifuge for 1–2 hours. After removal of the supernatant the deposit on the walls of the tubes was washed down with 2 c.c. saline, and this emulsion inoculated into the rabbit. The first inoculations were intravenous, but on account of a tendency for the virus emulsion to flocculate, and in view of Parker and River's (1936) results with similar preparations of vaccinia, it was thought advisable to give the three further weekly injections of similar material intraperitoneally. The serum (D63) was obtained one week after the last inoculation.

The antivaccinial serum S2 was kindly given us by Dr. E. V. Keogh. It was known to be highly active against the homologous strain (Keogh, 1936).

Titration Methods.

All the titrations were carried out on the chorio-allantoic membrane of the developing egg according to the methods described in earlier papers from this laboratory (Burnet, 1936 ; Keogh, 1936). Experience showed that stock emulsions of virus were best prepared by grinding a typical 3-day membrane which had been kept for a short period (1–7 days) in glycerol-Ringer solution with 5 c.c. of broth. After centrifugation the supernatant fluid is usually found to give a countable number of foci in the presence of normal serum when 0·05 c.c. of $1 : 10^4$ dilution is inoculated. Once preliminary experiments had indicated the degree of reduction by immune serum which was to be expected, dilutions of virus were chosen such as to give reasonable numbers of foci with normal and immune sera ; usually 5 or 6 eggs were inoculated from each mixture.

Virus dilutions were made in ice-chilled broth and added to equal volumes of undiluted normal or immune sera. Mixtures were left in the dark at room temperature for 2–3 hours before inoculation. Eggs at the 12th day of incubation were used, and after inoculation were incubated at 39°–39·5° C. for 3 days. Both viruses usually provide large distinct foci without any development of secondary foci ; irregularities of distribution and variable susceptibility of different eggs present the same problems as have been discussed in the papers referred to above. The canary virus lesions are opaque, rather conical elevations of the ectoderm, 1–2 mm. in diameter, while the fowl-pox lesions are larger and relatively flat with vaguely defined shelving edges.

TABLE I.—*Summary of Cross-neutralization Tests with Canary Virus and Fowl-pox.*

Virus.	Normal serum control.	Immune serum.	Per cent. "survivors".
Fowl-pox Antiserum :			
F.P. 4660	$3·2 \times 10^6$	$1·5 \times 10^5$	4·7
F.P. 4952	$4·0 \times 10^6$	$2·6 \times 10^5$	6·5
C.V. 4957	$3·6 \times 10^6$	$6·7 \times 10^5$	18
C.V. 5165	$1·1 \times 10^6$	$1·2 \times 10^5$	11
Canary Virus Antiserum :			
C.V. 5006	$2·6 \times 10^5$	$1·2 \times 10^4$	4·6
C.V. 5164	$1·8 \times 10^6$	$6·1 \times 10^4$	3·4
F.P. 5138	$6·8 \times 10^6$	$5·0 \times 10^5$	7·4
F.P. 5579	$2·2 \times 10^6$	$2·0 \times 10^5$	9·0
Vaccinia	$4·6 \times 10^7$	$5·5 \times 10^7$	100

The figures given under "Normal serum control" represent the number of virus particles calculated (on the basis of one focus representing one virus particle) to be present in 1 c.c. of a mixture of equal parts of undiluted virus and normal serum.

The figures under "Immune serum" are similarly calculated, and on the assumption that the "percentage law" of Andrewes and Elford holds. They may be taken to represent the number of virus particles present in 1 c.c. of the mixture of virus and immune serum which remain capable of initiating a specific lesion. The term "survivors" is used for these virus particles without any necessary implication that the remainder have been killed by the immune serum.

Experimental Results.

In addition to a number of preliminary tests two complete experiments have been done with each virus and each serum. Table I combines the results of all these complete experiments, the figures given being the number of active particles per c.c. of the original stock virus emulsion as deduced from the average pock count obtained with normal and with immune serum. The activity of the immune serum can be judged by the percentage of foci produced in terms of the control counts with normal serum.

TABLE II.—*Neutralization by Fowl-pox Antiserum H23.*

Virus.	Serum (undiluted).	Virus dilutions. 1 : 100.	1 : 1000.	1 : 5000.	1 : 10,000.	Equivalent titre.
Fowl-pox 4660	Immune H23	115, 90, 80 74, 64, 40 (77)	19, 12, 9 5, 1, 0 (7·8)	—	—	$1·5\times10^5$
—	Normal H25	—	—	52, 48, 38 28, 23, 6 (32·5)	—	$3·2\times10^6$
Canary virus 4957	Immune H23	—	53, 36 33, 27 21 (34)	—	6, 5, 2, 0 0 (2·6)	$6·7\times10^5$
—	Normal H25	—	279, 210 ± 208, 140 + 72 (182)	—	41, 27, 6 3† (19)	$3·6\times10^6$

TABLE III.—*Neutralization by Canary Virus Immune Serum.*

Virus.	Serum (undiluted).	Virus dilutions. 1 : 100.	1 : 1000.	1 : 500.	1 : 10,000.	Equivalent titre.
Canary virus 5164	Immune D63	48, 33, 10 † (30·3)	—	—	—	$6·1\times10^4$
—	Normal rabbit	—	112, 105 + 51, 3* (89)	—	10, 4, 4, 3 (5·2)	$1·8\times10^6$
Fowlpox 5138	Immune D63	—	41, 30, 28 14, 12 (25)	—	—	$5·0\times10^5$
—	Normal rabbit	—	—	112, 73 72, 55, 30 (68)	—	$6·8\times10^6$

Notes on Tables II and III.—Numbers represent counts of specific foci on each membrane; †, early death of egg. Bracketed figures are averages from which the equivalent titres are calculated. All counts are included for averages except one marked *, which must represent either a technical error or an extremely insusceptible egg.

Tables II and III provide the data from which Table I was constructed, but so as to avoid making the tables too unwieldy only one experiment of each group is included.

The results require very little comment. They are typical of all the serological results obtained when two similar but not completely identical antigens

are compared. In both cases the antiserum inactivates the homologous virus to a slightly higher degree than the heterologous.

As a check on the unlikely possibility that the inactivating effect of canary virus immune serum prepared in the rabbit might be due to antibodies developed in response to embryo antigens, this serum was tested against vaccinia and infectious laryngo-tracheitis viruses, both grown on the egg-membrane by the same method. In neither case was any inactivation evident. It is generally recognized that vaccinia is quite distinct from the bird-pox viruses, but it seemed advisable to determine whether any cross-neutralization could be detected by the present method. Table IV shows that no such effect was observed.

TABLE IV.—*Cross-neutralization with Vaccinia Virus.*

Virus.	Serum.	Virus dilution 10^4.	Equivalent titre.
Vaccinia	Canary virus immune	31, 24†	$5{\cdot}5 \times 10^7$
	Control	29, 17+	$4{\cdot}6 \times 10^7$
	Fowl-pox immune	150, 82, 10+, †	$1{\cdot}6 \times 10^8$
	Control	113, 92, 43, 13	$1{\cdot}3 \times 10^8$
Canary virus	Vaccinia immune	94, 82, 69, 61	$1{\cdot}6 \times 10^8$
	Control	91, 41+, 29, 27	$9{\cdot}4 \times 10^7$

DISCUSSION.

In previous work it has been shown that the viruses of infectious laryngo-tracheitis (Burnet, 1936), ectromelia (Burnet and Lush, 1936) and vaccinia (Keogh, 1936) can be effectively titrated on the chorio-allantoic membrane. The present work indicates that bird-pox viruses can be titrated in the same way. A study of Table II will show that despite a rather wide range of counts at each dilution, the average counts show an accurate linear relationship between concentration of virus and pock count. The fact that this holds equally well with immune sera indicates that the "percentage law" of Andrewes and Elford (1933) is applicable to these neutralizations also.

The demonstration of the close serological relationship of these two strains of virus, which produce superficially very distinct lesions on two different host species, could hardly have been made by any other technique, and certainly not with the same degree of accuracy. If the canary-pox and fowl-pox are serologically almost identical, it is more than likely that all bird-box strains are similarly related. The more important question of the relationships between the various mammalian pock diseases may perhaps also be susceptible of investigation along these lines. Vaccinia virus grows excellently on the egg-membrane, and Torres has described positive results with a strain of alastrim. As far as we are aware pock diseases of other mammals have not been investigated. The advantages of having a common susceptible organism for the whole group are so obvious that it is to be hoped that investigators having access to material from cases of smallpox and of the more important animal pock-diseases, none of which are available in Australia, will explore the possibilities of the method used in the present study.

SUMMARY.

1. The viruses of fowl-pox and Kikuth's canary disease can be accurately titrated by the pock-counting method on the chorio-allantoic membrane.

2. Cross-neutralization tests indicate that the two viruses, though not identical, are serologically very closely related.

3. There is no cross-reaction with vaccinia virus.

REFERENCES.

ANDREWES, C. H., AND ELFORD, W. J.—(1933) *Brit. J. exp. Path.*, **14**, 367.
BURNET, F. M.—(1933) *J. Path. Bact.*, **37**, 107.—(1936) *J. exp. Med.* (in the press).
Idem AND LUSH, D.—(1936) *J. Path. Bact.* (in the press).
GAEDE, H.—(1935) *Zbl. f. Bakt.*, Abt. I, Orig., **135**, 342.
HERZBERG, K.—(1933) *Ibid.*, **130**, 326.—(1934) *Ibid.*, **131**, 358.
KEOGH, E. V.—(1936) *J. Path. Bact.* (in the press).
KIKUTH, W., AND GOLLUB, H.—(1932) *Zbl. f. Bakt.*, Abt. I, Orig., **125**, 313.
PARKER, R. F., AND RIVERS, T. M.—(1936) *J. exp. Med.*, **63**, 69.
TORRES, C., AND TEIXEIRA, J. DE C.—(1935) *C.R. Soc. Biol.*, Paris, **118**, 1023.

A NOTE ON THE GUINEA-PIG METHOD OF ASSAY OF LIVER EXTRACT.

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IN a recent study of the guinea-pig method of testing liver extract for its hæmatopoietic activity, Ågren and Caspersson (1936) describe results which appear in marked disagreement with those of the present author. The above investigators have apparently overlooked a detailed account of the guinea-pig test (Jacobson, 1935), and hence do not mention the method of housing and the diet of their animals, the selection of animals with low and stable reticulocyte levels, or the selection of reactive guinea-pigs, nor do they conform to the author's observation period of six successive days or to the method of estimation of reticulocytes.

In Table I are reproduced the data of Ågren and Caspersson, representing average values of percentage of reticulocytes in a group of 25 untreated guinea-pigs and in a group of 25 animals injected with liver extract. For comparison there are presented in Table II averages of percentage of reticulocytes of a group of 5 guinea-pigs which responded *negatively* to the administration of

various materials, and of a group of 6 animals which responded *positively* to the administration of certain liver extracts. These latter data are taken from previously published values (Jacobson, 1935, Table III, p. 668).

TABLE I.—*Data of Ågren and Caspersson.*

(P.c. reticulocytes.)

	Day.							
	1.	3.	5.	7.	9.	11.	13.	15.
Untreated	0·61	0·67	0·86	1·47	1·29	1·15	1·16	1·38
Liver-treated	0·78	0·76	0·79	1·65	1·28	1·53	1·34	1·39

TABLE II.—*Data of Jacobson* (1935).

(P.c. reticulocytes.)

	Day after administration.						
	0.	1.	2.	3.	4.	5.	6.
Negative	0·8	0·9	1·0	1·1	1·0	0·9	1·0
Positive	0·9	1·1	1·5	**2·3**	**2·2**	**2·3**	**2·2**

It is apparent from the accompanying tables that in neither group of Ågren and Caspersson's guinea-pigs did the percentage of reticulocytes reach a positive response level (of 2·0 p.c. or over on two successive days of a 6-day period) that is shown in Table II. According to the present author's definition (Jacobson, 1935), the liver-treated guinea-pigs of Ågren and Caspersson exhibited a non-reactive state. The explanation of the fluctuating values in the untreated group (compare data of first groups in Tables I and II) may be dual. In the first place, the values may be representative of a group of guinea-pigs which were not selected for low and stable reticulocyte levels. In the second place, it is very probable that part of the fluctuating reticulocyte levels can be ascribed to hæmorrhage, since Ågren and Caspersson state that for each reticulocyte count as much as 0·010 to 0·020 c.c. of blood were removed—an amount that may readily induce a slight reticulocytosis.

The above authors are not apparently cognizant of the extensive control data that have been described, on the quantitative relation between dosage of administered liver extract and response, and of the negative effects of many materials, including the extracts of pernicious anæmia liver, gastric juice, and sources of extrinsic factor (Jacobson, 1935; Miller and Rhoads, 1935).

REFERENCES.

ÅGREN, G., AND CASPERSSON, T.—(1936) *Brit. J. exp. Path.*, **17**, 87.
JACOBSON, B. M.—(1935) *J. clin. Invest.*, **14**, 665; *ibid.*, **14**, 679.
MILLER, D. K., AND RHOADS, C. P.—(1935) *New Engl. J. Med.*, **213**, 99.

THE TYPHUS GROUP OF DISEASES IN MALAYA. PART VI: THE SEARCH FOR CARRIERS.

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THE principal carriers of the typhus-like fevers throughout the world may be regarded as falling into four main groups—body-lice, ticks, rat fleas and larval mites. Extensive examinations of healthy and sick labourers in areas in which rural typhus was endemic failed to discover a single body-louse (Gater, 1930); school-children are free from them; patients suffering from any of the three forms of the Malayan typhus fevers are nursed in the general wards of hospitals without transference of infection to others. It is certain, therefore, that the body-louse plays no part in the transmission of any of these three fevers. Ticks are known to be the carriers of typhus-like fevers in North and South America, South Africa and Europe. Rat fleas are carriers of other members of the typhus group in America and the Mediterranean zone. In Part IV of this series of papers it has been noted that the available epidemiological evidence incriminates two larval mites, *Trombicula akamushi* and *Trombicula deliensis*, as carriers of rural typhus and the tsutsugamushi disease in Malaya. Gater, as a result of his investigations in endemic areas, inclines to the view that these two mites, respectively the carriers of the tsutsugamushi disease in Japan and the closely akin (if not identical) mite-fever of Sumatra, are merely forms of the same species. Further, he thinks that this species may prove to be the carrier in Malaya of both rural typhus and the tsutsugamushi disease, two diseases that retain their classification as separate entities on a single clinical distinction, being identical in all other features. The breeding of these trombidiids through their life-cycle, in quantities sufficient for the carrying out of transmission experiments, presents many difficulties; and much preliminary work will be necessary before such experiments become possible.

The search for a carrier of any of the members of the typhus group of diseases may become complicated to an unforeseen degree as it progresses. Many carriers may be found, one being of primary importance, the others of secondary importance, as regards prevalence in nature and the virulence of the virus transmitted. The present investigation was restricted to the *rôle* of two species of tick and one species of flea as carriers of rural and urban typhus. The sources of virus were guinea-pigs infected with the laboratory strains of the two diseases.

THE *RÔLE* OF THE RAT FLEA IN THE TRANSMISSION OF RURAL AND URBAN TYPHUS.

The Breeding of Fleas.

The species of flea used was the rat flea, *Xenopsylla cheopis* (Roths.), of which a strain had been established in the year 1931 with the progeny of fleas taken from local rats. The fleas were fed on local brown mice, the technique employed being similar to that described by Leeson (1932). Later it was found that white mice were in many respects more suitable, and the use of brown mice was discontinued.

Transmission by Feeding.

Procedure.—The procedure was modelled on that adopted by Dyer and his associates (1931) in their demonstration of the transmission of the endemic typhus of the United States of America by the rat flea. Only fleas freshly emerged from the pupal stage were used ; none of them had previously fed on other animals. As a preliminary, from the batch of fleas destined for this investigation, 12 were ground in sand, and a suspension in saline injected intra-peritoneally into 2 guinea-pigs, and intra-ocularly into 2 rabbits. The guinea-pigs showed no febrile or scrotal reaction ; the rabbits showed no ocular reaction, and the Weil-Felix reaction remained negative during 60 days' observation. It was clear, then, that the fleas to be used were free from infection with the viruses of rural and urban typhus. In the test proper two series of experiments were carried on simultaneously, each the exact duplicate of the other in procedure and technique, the virus being of the urban typhus strain in the one, and of the rural typhus strain in the other. Below are summarized in stages the procedure and results in the case of the urban typhus strain, in which success was attained.

Transmission of urban typhus.—Successively, at 3-day intervals, 3 white rats were inoculated intra-peritoneally with brain emulsion of a reacting guinea-pig of the " Manickam " strain. The intervals between inoculations ensured a supply of infected blood for as long a period as possible. Since the infection of the white rat with strains of either type of tropical typhus is " inapparente ", 2 control guinea-pigs were inoculated at the same time as each rat. After inoculation each rat was placed in a box of dimensions 24 in. × 16 in. × 16 in., with the base and edges of copper, sides and ends of glass, and with a top of fine wire gauze intersected by a broad copper strip, in which was cut a hole fitted with a lid. During the second 3-day interval 75 fleas were introduced into the box, and were allowed to feed on the white rats for 15 days.

After the 15-day period of feeding 38 fleas were transferred to a second similar box ; 7 fleas were retained in a mouse-jar, together with a white mouse, for subsequent search for *Rickettsia* in smears ; 13 fleas were ground, suspended in saline, and injected intra-peritoneally into 4 male guinea-pigs. All 4 guinea-pigs, after the usual incubation period, reacted with fever and scrotal swelling ; 2 of them, on inoculation during convalescence with passage virus, proved to be immune ; while, conversely, 2 convalescent guinea-pigs of the passage urban strain proved to be immune to re-infection with infected material from this flea strain. On the same day that the fleas were withdrawn from

the first box, the 3 white rats on which the fleas had fed were removed, killed, and a suspension of each brain inoculated into a male guinea-pig, and a mixed emulsion of all three brains into a fourth male guinea-pig. All 4 guinea-pigs reacted with typical fever and scrotal swelling.

At this stage of the experiment, therefore, proof was obtained that the white rats had all been successfully infected with the virus of urban typhus, and that the fleas had acquired from these rats, by feeding, a virulent infection of the urban type.

Into the second box, to which the 38 fleas had been transferred, were placed 3 normal white rats. Since the infection of white rats with the virus of tropical typhus is " inapparente ", it was considered advisable to kill the three rats at intervals, in order to ensure demonstration of the virus in one rat at least, should infection have occurred at all. The first rat was killed after 9 days' contact with the infected fleas, and brain and spleen suspensions separately inoculated into a pair of guinea-pigs ; none of the 4 reacted. The same procedure was followed in the case of the second rat after 11 days' contact with the fleas ; one of the 4 guinea-pigs inoculated reacted after the usual incubation period with both fever and scrotal swelling. The third rat was killed after 14 days' contact with the fleas ; all 4 guinea-pigs inoculated reacted with fever and scrotal swelling ; 2 of these proved to be immune on reinoculation with passage virus of the urban strain, while 2 convalescent guinea-pigs of the passage strain proved to be immune to reinfection with material from guinea-pigs of this flea-white-rat strain. Thus successful transmission was demonstrated.

Smears of the mid-guts of those fleas retained on the white mouse for 19 days showed innumerable intra-cellular and extra-cellular *Rickettsia* morphologically identical with *R. prowazeki*. Smears of normal fleas showed no *Rickettsia*.

The supply of white rats used in this investigation had all been derived from one male and two females. It has been demonstrated repeatedly by the authors that the offspring of rabbits and guinea-pigs born during infection with, or convalescence from, tropical typhus are not immune to reinfection, but are as susceptible as control animals. Nevertheless it was thought unwise to rely on these observations for assurance that the stock of white rats was free from infection with tropical typhus ; and the brains of 2 uninoculated rats from stock were injected intraperitoneally into 4 guinea-pigs. None showed any trace of infection.

To confirm the above success the experiment was repeated, the only difference in procedure being that the fleas, on removal from the first box, were retained for 24 hours in a measuring cylinder for the deposition of fæces before being transferred to the second box, and that the number of days' contact between fleas and rats in the second box differed. Successful infection of fleas and transmission by them was again achieved, the virus being transmitted to 1 of 3 white rats. The rat thus infected had been in contact with the fleas for 13 days ; the 2 rats that escaped infection, for 14 days. As before, the immunological identity of the flea-white-rat strain with the parent passage strain of urban typhus was demonstrated.

In each experiment rats and glass boxes were examined for the presence of any blood-sucking parasite other than the rat flea ; none was found.

Attempts to transmit rural typhus.—In the case of the parallel series of experiments carried out *pari passu* with the above series, in which the virus used was of the "Seerangayee" strain of rural typhus, all test inoculations of fleas and of material from white rats gave negative results in guinea-pigs and rabbits; so that no evidence was forthcoming that the rat flea could transmit or acquire the virus of rural typhus under experimental conditions.*

Transmission of Urban Typhus by the Application of Flea-Virus to Scarified Skin.

The above investigations were extended to determine possible methods of transmission; in particular, whether it is possible to transmit the virus of urban typhus by rubbing either fæces from infected fleas or crushed infected fleas into scarified skin. Transmission of the endemic typhus of North America by these two methods has been demonstrated under experimental conditions by Dyer and his associates (1932*a*).

Transmission by fæces.—Into a 500 c.c. measuring cylinder, having a disc of filter-paper at the bottom, 26 infected fleas (*X. cheopis*) were placed for 24 hours for deposition of fæces. From the filter-paper the fæces were collected, and suspended in ½ c.c. of saline. For inoculation 4 male guinea-pigs were selected, the dorsal flanks were shaved, and lightly scarified with the point of a needle. Into the abraded surface one drop of the fæcal suspension was rubbed with a glass rod. Two of the 4 guinea-pigs showed neither febrile nor scrotal reaction during 22 days' observation, but on reinoculation after 28 days with passage virus were found to be immune. The other 2 guinea-pigs showed no reaction during 12 and 14 days' observation respectively, after which period they were killed, and a suspension of each brain separately injected intra-peritoneally into 2 fresh guinea-pigs. One guinea-pig of each pair reacted with typical fever and scrotal swelling after an incubation period of 9 days, and one of these proved to be immune on test inoculation during convalescence with passage virus. The third guinea-pig reacted with typical fever; the fourth failed to react.

Transmission by crushed fleas.—Four infected fleas were crushed in a mortar in ½ c.c. of saline. The skin of 4 male guinea-pigs was scarified, and the suspension of crushed fleas applied. Two of the guinea-pigs failed to show febrile or scrotal reaction during 22 days' observation, but on test inoculation with passage virus during convalescence were found to be immune. The third scarified guinea-pig showed no reaction during 14 days' observation, and was then killed and a suspension of brain inoculated intra-peritoneally into 2 fresh guinea-pigs. A short febrile reaction developed in both after an incubation period of 11 days, and on test inoculation with passage virus one month later both proved to be immune. The fourth scarified guinea-pig developed a febrile reaction on the 9th day after inoculation. Fever was continuous until the 12th day, when the temperature fell to normal; on this day the animal was killed, and a suspension of brain injected intraperitoneally into 2 fresh

* One of us (Savoor) has since repeated by a modified method this attempt to transmit the virus by fleas, and has obtained evidence which indicates that, under experimental conditions, transmission of the virus of rural typhus from guinea-pig to guinea-pig is possible. It may be that the rat flea will prove to be a carrier of secondary importance. Investigations are being continued.

guinea-pigs. One of these died, afebrile, on the 10th day after inoculation; the other reacted with fever on the 10th and 11th days, but the temperature fell to normal on the 12th day, and passage of a suspension of its brain into a third generation of guinea-pigs failed to evoke reaction.

The foregoing experiments suggest that in nature a possible method of transmission of the virus may be the inoculation of the crushed tissue of, or the fæces of, infected fleas into abrasions caused either by the bite of the flea or by scratching.

The Multiplication of the Rickettsia *of Urban Typhus in* X. Cheopis.

Two guinea-pigs inoculated with passage virus of the "Manickam" strain of urban typhus were placed on the first day of the febrile reaction into a glass box (box A), together with 140 freshly-hatched fleas. Nine days later as many fleas as could be recovered (68 in number) were removed from the box and from the guinea-pigs. They were placed in a measuring cylinder, and starved for 24 hours in order to eliminate in some degree infectivity due to fæces. Two of the fleas were then dissected, the mid-guts smeared and stained by Giemsa's method. Innumerable *Rickettsia* were seen, morphologically identical with *R. prowazeki*. Six fleas were stunned by ether, washed in saline, and ground. Twelve c.c. of saline were added, and the suspension centrifuged at low speed to get rid of flea *débris*. The supernatant clear fluid was used for titration experiments. Four inocula were prepared, each one a dilution equivalent to 1 flea, 1/10, 1/100 and 1/1000 of a flea respectively; they were severally inoculated intraperitoneally into pairs of guinea-pigs. The latter all reacted with fever; 2 males that received 1 flea and 1/10 of a flea reacted with scrotal swelling also. The 2 guinea-pigs that received 1/1000 of a flea were later reinoculated with passage virus; one died on the 10th day without rise of temperature, and the other proved to be immune. It was evident, therefore, that 1/1000 part of a flea was infectious to guinea-pigs. The remaining 60 fleas were put into three glass boxes, B, C and D, 20 into each. Into box B was placed a guinea-pig on the first day of its fever due to infection with passage virus of urban typhus; into box C was placed a normal guinea-pig, and into box D a guinea-pig convalescent from infection with urban typhus.

Nine days later the fleas were removed from these three boxes. Two fleas from each box were dissected, the mid-guts smeared, stained, and examined for *Rickettsia*; none was found. Six fleas from each box were ground as before and suspensions were prepared, equivalent to 1, 1/10, 1/25, 1/100, 1/250, 1/1250, 1/2000, 1/5000 of a flea. The guinea-pigs inoculated with the equivalent of 1 flea and 1/10 of a flea developed typical febrile reactions; those that had received 1/10 of a flea were reinoculated with passage virus during convalescence, and found to be immune. Those guinea-pigs injected with inocula equivalent to 1/25 of a flea and less did not develop a febrile reaction, and on reinoculation with passage virus were found to be non-immune.

The fleas used came from a stock bred in the laboratory for three years, and proved by inoculation into laboratory animals to be free from infection with tropical typhus. The above results show that the fleas became infected to such a degree that, in 10 days' time, as little as 1/1000 part of a flea was

infective to guinea-pigs. We consider this degree of infectivity to be due to the multiplication of the virus within the flea, not to mere survival of ingested virus, for the following reasons. Since the blood of guinea-pigs infected with the virus of urban typhus is infective during fever only, it is probable that the fleas got infective feeds for the first 6 days, and that no infected blood was ingested from the 7th to the 9th days. During the 6th to the 10th days the infected blood previously ingested would have been voided. Again, a batch of the infected fleas, when further fed on infected guinea-pigs in box B under identical conditions, showed a marked diminution in infectivity.

Inasmuch as titration showed the infectivity of the fleas from boxes B, C and D to be the same (1/10 dilution) after the lapse of 20 days from the original infecting feed in box A, although from the 11th to the 19th day these boxes contained respectively an infected, a normal, and a convalescent guinea-pig, it would appear that in nature the infectivity of infected fleas is not materially affected by the state of infection, susceptibility, or insusceptibility of the host.

It is apparent from these experiments that the *Rickettsia* of urban typhus do not multiply in *X. cheopis* to as great a degree as do the *Rickettsia* of Mexican-American typhus. Dyer *et al.* (1932*b*) found that, 20 days after an infective feed, 1/64,000 part of a flea was infective to guinea-pigs ; whereas our results showed that by that time the infectivity of fleas infected with urban typhus had passed its maximum and was declining rapidly. Dyer *et al.* used infected rats as a source of virus, whereas we used guinea-pigs, but it is very improbable that this factor accounts for the difference noted. It is possible that this is due to a greater degree of parasitism exhibited by *R. mooseri* to *X. cheopis*. Mexican typhus, maintained in nature by the rat-flea-rat cycle, is considered to have existed for centuries. Tropical typhus, on the other hand, is considered by Fletcher (1925) to have been only recently introduced into Malaya. If this view be correct, then it may be that the difference lies in the length of adaptation of the respective *Rickettsia* to the rat flea.

The Non-Survival of the Virus of Urban Typhus through the Egg of the Rat Flea (X. cheopis).

When, in the repeated successful transmission experiment noted above, the last white rat was removed from the second glass box, all fleas were also removed. For the next 9 days, daily search failed to discover any adult flea in the box ; only larval forms were seen. On the 10th day adult fleas appeared ; they were ground in saline, and the suspension injected intra-peritoneally into 2 guinea-pigs. Both failed to react. On the 11th day another batch of newly hatched adults was secured from the box and similarly injected into two other guinea-pigs ; again no reaction occurred. Thus the progeny of infected fleas proved to be free from infection with the virus.

THE *RÔLE* OF TICKS IN THE TRANSMISSION OF URBAN AND RURAL TYPHUS.

The ticks subjected to experiment were of two species, the American wood tick, *Dermacentor andersoni*, and the dog tick, *Rhipicephalus sanguineus*. *Dermacentor andersoni*, which, so far as is known, is not found in Malaya, is the most important carrier of Rocky Mountain spotted fever, and was used at

the suggestion of Dr. R. R. Parker, of the Rocky Mountain Spotted Fever Laboratory, Hamilton, United States of America, in the hope that it might prove, under laboratory conditions, a carrier of one or other of the typhus diseases of Malaya, and so enable such virus to be despatched to America for comparison with the large number of typhus strains there available. *Rhipicephalus sanguineus*, the commonest local tick, is the most important vector of the " fièvre boutonneuse " of the Mediterranean littoral, and of African tick typhus. Engorged ticks taken from a dog were allowed to oviposit, and the larvæ (and subsequent stages) were fed on guinea-pigs in the manner described by American workers on Rocky Mountain spotted fever (*vide infra*).

Procedure.

The procedure adopted was identical in experiments with both species of tick; it had been successful in the hands of Spencer and Parker (1923) in the case of *Dermacentor andersoni* and Rocky Mountain spotted fever, being based on the phenomenon of " reactivation " described by these workers.

The uninfected ticks, nymphs or adults were fed in metal capsules, as recommended by Jellison and Philip (1933), on infected guinea-pigs of the rural or urban strain of tropical typhus from the first or second day of fever onwards for 3 or 4 days. They were then removed, being partially engorged, and kept for periods varying from 2 to 10 weeks, some at room temperature, some at a temperature of 3° to 6° C., this period of time being regarded as adequate for any virus ingested to multiply and become generalized throughout the tick tissues. The ticks were then allowed to feed on uninfected guinea-pigs for 3 days, after which they were removed, and ground in sterile sand and saline. The resulting suspension was centrifuged, and the supernatant fluid injected intra-peritoneally into two normal guinea-pigs. The guinea-pigs on which the ticks were re-fed, and those into which the tick emulsions were injected, were observed for 28 days for development of fever and scrotal swelling. They were given test inoculations of the respective passage virus 1 to 3 months later.

Results.

No clear-cut positive reactions have been obtained with either species of tick in the case of either strain of tropical typhus.

Rural typhus: Dermacentor andersoni.—About 40 nymphs and adults were fed in batches on 6 infected guinea-pigs. They were re-fed, some after an interval of 4 weeks, others after 8 weeks, on 3 normal guinea-pigs, and then injected as a suspension into 6 guinea-pigs. The 3 guinea-pigs that served for re-feeding, and 5 of the 6 injected with the tick emulsion showed no trace of reaction; the 6th injected guinea-pig developed a delayed and very mild reaction, which conferred no immunity against subsequent inoculation of passage virus. All 9 reacted on test inoculation with passage virus, 6 dying of the infection.

Rural typhus: Rhipicephalus sanguineus.—Seventy nymphs and adults were fed on 3 infected guinea-pigs. They were re-fed after 15 days on 3 normal guinea-pigs, and then injected as a suspension intraperitoneally into 3 pairs of

guinea-pigs. All 9 test guinea-pigs remained afebrile. Eight were reinoculated with passage virus ; 1 died afebrile on the 18th day after inoculation, 7 showed typical febrile reaction. Six survived the infection, 3 of them being guinea-pigs on which ticks had been re-fed, and 3 having been originally inoculated intraperitoneally with tick tissues. Thus, by the criterion of occurrence of febrile reaction, the guinea-pigs were still susceptible ; but the low mortality (1 out of 7), compared with the usual mortality of more than 90 p.c. in passage animals of this strain, suggests that a slight degree of immunity had been conferred, and that a feeble and " inapparente " infection may have been acquired and transmitted by the ticks. Possibly such an infection may have been due to mere survival of virus ingested by the ticks from the passage animals used as the source of virus.

Urban typhus : Dermacentor andersoni.—Twenty-four nymphs and adults were fed in batches on 8 infected guinea-pigs of the " Manickam " strain. They were re-fed in two batches on 2 fresh guinea-pigs after intervals of 5 and 10 weeks respectively, and then injected as an emulsion into 4 male guinea-pigs. All 6 guinea-pigs failed to show reaction, either febrile or scrotal. Four of the 6 were reinoculated with passage virus ; 1 of them failed to react, the other 3 reacted typically.

Urban typhus : Rhipicephalus sanguineus.—Seventy nymphs and adults were fed in three batches on 3 infected guinea-pigs of the " Manickam " strain. Two batches were re-fed on 2 fresh guinea-pigs after 2 weeks, a third batch on 1 fresh guinea-pig after 3 weeks. The ticks were then ground, and a suspension in saline injected into 6 male guinea-pigs. Of the guinea-pigs that served for re-feeding, 2 showed no trace of febrile or scrotal reaction. The third (a female) developed a febrile reaction from the 8th to the 11th day ; it was killed on the 11th day and the brain inoculated into 2 male guinea-pigs, both of which failed to show any febrile or scrotal reaction. All 6 guinea-pigs injected with tick emulsion failed to show any reaction. They, together with 2 on which the ticks had re-fed, were reinoculated with passage virus ; 7 reacted typically, 1 failed to react.

To summarize, more than 200 ticks ingested infected blood, and, at varying intervals, were re-fed on, or their ground tissues were injected into 33 fresh guinea-pigs. Thirty-one of these showed no trace of reaction ; 2 showed a mild febrile reaction, which, in one case, could not be reproduced on passage, and in the other case conferred no immunity. Test inoculations with passage virus were made into 28 of the 33 guinea-pigs ; 26 reacted, 2 failed to react. The only evidence suggesting that the ticks may have transmitted or acquired either of the viruses was obtained in the case of rural typhus, in the appreciably diminished mortality of the experimental guinea-pigs following test inoculation with passage virus, as compared with the mortality in passage animals of this strain. On this evidence one cannot exclude the possibility that ticks may play a minor *rôle* in the epidemiology of rural typhus. Further, in connection with rural typhus, the following points are to be noted : (*a*) that the experimental animal used was the guinea-pig ; (*b*) that attempts to infect guinea-pigs with human virus succeed only in 6–11 p.c. (*vide* Part I of this series) ; (*c*) that the " Seerangayee " guinea-pig strain (of human origin) used in the above experiments in attempted infection of ticks is very virulent for guinea-pigs,

failure to react being extremely rare and mortality being 90 p.c. or more. It is conceivable that the insusceptibility of the guinea-pig to infection with the virus of rural typhus by ticks—whether by feeding or by injection of tick tissue—may have been due to a reversion of the virulence of the " Seerangayee" strain, consequent on being passed through the tick, to the lower level typical of strains of human origin.

SUMMARY.

1. Transmission of the virus of urban typhus under experimental conditions from rat to rat by the rat flea (*X. cheopis*) by feeding has been effected. Collateral attempts to transmit the virus of rural typhus by precisely the same procedure failed.

2. Transmission of the virus of urban typhus was also achieved by the inoculation of fæces or crushed tissue of infected fleas into the scarified skin of guinea-pigs.

3. Multiplication of the virus of urban typhus occurs within the rat flea.

4. Infection with the virus of urban typhus is not hereditary in the rat flea.

5. Attempts to transmit the virus of urban or rural typhus by two species of ticks failed. In the case of rural typhus a lessened mortality in the experimental guinea-pigs following test inoculation with passage virus makes it, however, difficult to exclude ticks entirely as a minor factor in the epidemiology of rural typhus.

We wish to thank Dr. R. R. Parker, Director of the Rocky Mountain Spotted Fever Laboratory, Montana, for valuable advice regarding the experimental infection of ticks ; and Dr. J. A. Chelliah, of this Institute, for his help in various phases of this investigation.

REFERENCES.

DYER, R. E., CEDER, E. T., LILLIE, A., *et al.*—(1931) *Publ. Hlth. Rep. Wash.*, **46**, 2481.

DYER, R. E., CEDER, E. T., WORKMAN, W. G., *et al.*—(1932*a*) *Ibid.*, **47**, 131.—(1932*b*) *Ibid.*, **47**, 987.

FLETCHER, W., AND LESSLAR, J. E.—(1925) *Bull. Inst. Med. Res. F.M.S.*, No. 2 of 1925, p. 27.

GATER, B. A. R.—(1930) *Trans. Far Eastern Ass. Trop. Med.*, **2**, 132.

JELLISON, W. L., AND PHILIP, C. B.—(1933) *Publ. Hlth. Rep. Wash.*, **48**, 1081.

LEESON, H. S.—(1932) *Bull. ent. Res.*, **23**, 25.

LEWTHWAITE, R., AND SAVOOR, S. R.—(1936) *Brit. J. exp. Path.*, **17**, 1 and 23.

SPENCER, R. R., AND PARKER, R. R.—(1923) *Publ. Hlth. Rep. Wash.*, **38**, 333.

THE PURIFICATION OF STAPHYLOCOCCUS TOXOID.

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STAPHYLOCOCCUS toxoid consists essentially of the toxic filtrate of a staphylococcus culture, to which has been added 0·3 p.c. formalin. It has been found by the author that the toxin can be concentrated as follows: It is saturated with ammonium sulphate, centrifuged, the supernatant discarded and the precipitate redissolved in water. By this process 90–98 p.c. of the toxin is recovered. So concentrated and partially purified it has been found to be more stable than raw unconcentrated material. Furthermore, its antitoxin-binding and hæmolysing powers remain constant for many months, indicating that very little or no autotoxoiding action had taken place.

For the routine preparation of toxoid, the toxin-formalin mixtures were incubated at 37° C. until toxin-free, as indicated by the absence of hæmolysing power for rabbit's red corpuscles. During the process of toxoiding, however, a considerable loss of antigen occurred; as indicated by antitoxin-binding estimations, by the method of Dolman and Kitching (1935). A similar loss of antigen has been reported by Burnet (1932), and Dolman and Kitching (1935). When the present investigation was begun, it was not clear whether such antigen destruction (or apparent destruction) ceased when the toxoiding process was completed. The loss of antigen might have been caused by the formalin used, or by some substance or substances produced by the staphylococci. On the assumption that the disappearance of antigen did not cease when toxoiding was complete, an attempt was made to purify and stabilize toxoid by the method successfully used in the case of toxin.

The present communication reports experiments designed, (*a*) to purify toxoid as ordinarily prepared, and (*b*) to produce toxoid free from the nitrogenous constituents of the medium.

PURIFICATION OF TOXOID WITH AMMONIUM SULPHATE.

Raw toxoid of known antitoxin-binding power was saturated with ammonium sulphate. The precipitate which formed was filtered through long cloth; the cloth and precipitate were placed in a cellophane bag, and dialysed against running tap-water until sulphate-free. This required two days. The volume of the "dialysee"* was noted, and its antigen content determined by the antitoxin-binding-power method. (All results recorded in this paper refer to *a*-antigen only (Glenny and Stevens, 1935) and the

* The term "dialysee" is introduced for convenience to denote the undialysable residue under the experimental conditions employed.

quantity of antigen is expressed in terms of equivalent international standard units of staphylococcus antitoxin.)

TABLE I.—*Recovery of Antigen on Purifying Raw Toxoid by Precipitating with Ammonium Sulphate.*

Sample.	Raw toxoid.		Purified toxoid.		Recovery (p.c.).
	Volume (c.c.).	Value (units per c.c.).	Volume (c.c.).	Value (units per c.c.).	
H. 18	1000	14	250	55	98
E. 171	800	9	300	23	95
E. 172	750	6	250	16	89
E. 282	400	16	130	40	81
E. 394	500	14	110	55	86
E. 398	1400	11	220	70	100

A consideration of the results, recorded in Table I, shows that the recovery of antigen was of a high order. Further experiments indicated that even by half saturation with ammonium sulphate, much of the antigen was precipitated. In one such experiment 60 p.c. was recovered from 400 c.c. of raw toxoid. These experiments demonstrated :

(*a*) That staphylococcus *a*-toxoid is completely precipitated by full saturation with ammonium sulphate.

(*b*) That this antigen does not pass through the dialysing membrane used, viz. commercial cellophane, grade 400.

(*c*) That the antigen of raw toxoid could be partially purified, concentrated and recovered quantitatively.

PREPARATION OF TOXIN ON A DIALYSED MEDIUM.

The routine medium used in these laboratories for the growth of staphylococci in the preparation of toxin is composed of peptone 1 p.c., beef-juice 1 p.c. and 3 p.c. of a buffer solution. The nutritional elements of this medium may be divided roughly into two classes, with large and small molecules respectively. These may be separated by two methods, (*a*) by saturation with ammonium sulphate, when the large molecules only are precipitated, and (*b*) by dialysis, where the small molecules pass the membrane, leaving behind the large ones.

It has been shown that staphylococci will grow on a semi-synthetic medium (Hughes, 1932 ; and Knight, 1935). This suggested that staphylococci might grow and produce toxin on the small molecule fraction of the routine medium, thus providing a means of producing toxoid free from all constituents of the medium. The " small molecule medium " could be prepared by dialysis ; the dialysate would be free from large molecules. Toxin produced on this could be freed from the medium by subsequent re-dialysis. Thus a final preparation could be made composed entirely of large molecular material derived from bacterial activity. Furthermore, instead of immediately dialysing the raw toxin, or toxoid, the antigen could be precipitated by ammonium sulphate, and the precipitate dialysed free from ammonium

sulphate as described above. This latter process, with the proposed new medium, would give the added advantage of a high volume concentration of antigen.

With the foregoing in mind the following experiments were carried out :

200 c.c. of 5 p.c. Parke Davis peptone, and 5 p.c. (by vol.) of Allen and Hanbury's beef-juice were boiled, adjusted to pH 6·5, with 10 p.c. KOH, and filtered hot. The filtrate was cooled, placed in a cellophane bag, and dialysed against 800 c.c. of tap-water containing 30 c.c. of concentrated phosphate buffer, pH 6·5. The buffer solution was prepared by adding to a 20 p.c. solution of K_2HPO_4, a 20 p.c. solution of KH_2PO_4, until the mixture diluted 1 : 100 had the required pH. Dialysis was carried out in the ice-chest for two days. The final volume of the dialysee was 250 c.c. and the dialysate was 750 c.c. *The dialysate contained no nitrogenous material precipitable by saturation with ammonium sulphate,* whereas the dialysee contained much. The dialysee was made up to 1 litre with a 3 p.c. solution of the buffer ; 750 c.c. of the diluted dialysee were used.

To each medium powdered agar was added, to give a concentration of 0·6 p.c. ; this was dissolved by steaming, after which the medium was distributed in 250 c.c. quantities placed in litre bottles. These were then autoclaved, and the medium allowed to set on the flat side of the bottles. Each bottle was inoculated with staphylococcus Strain Wood 46, and incubated in an atmosphere of 20 p.c. CO_2, and 80 p.c. O_2. The toxin yields are tabulated in Table II. The growth on the diluted " dialysee " medium was very poor, whereas on the " dialysate " it was good.

TABLE II.—*Toxin Yields on " Dialysee " and " Dialysate " Media.*

Dialysate.

Bottle No.	1.	Time	48	hours.	Toxin yield		20	units.
,, ,,	2.	,,	48	,,	,,	,,	20	,,
,, ,,	3.	,,	72	,,	,,	,,	50	,,

Dialysee.

Bottle No.	1.	Time	48	hours.	Toxin yield		5	units.
,, ,,	2.	,,	48	,,	,,	,,	5	,,
,, ,,	3.	,,	72	,,	,,	,,	10	,,

Staphylococci were similarly grown on a medium containing small molecules at normal concentration, and the large molecules at an unusually high concentration, namely " undiluted dialysee ". The yields of toxin on " dialysate medium " and on " undiluted dialysee medium " were usually identical, but the *growth* of microbes was much greater on the latter.

CHARACTER OF DIALYSATE, DIALYSEE AND ROUTINE MEDIA TOXINS.

The " hæmolytic ratios " (*i. e.* the number of hæmolysing units to one antitoxin binding unit) of toxins from " dialysed medium " have been found consistently to be one-third or lower than the ratios of toxins obtained on undialysed or undiluted " dialysee " media. Examples are shown in Table III.

TABLE III.—*Hæmolytic Ratios of Toxins Prepared on Different Media.*

Source of toxin.	Hæmolysing value (units).*	Antitoxin binding value (units).	"Hæmolytic ratio."
Routine medium.			
Sample E. 338	2000	25	80
,, E. 262	700	10	70
,, E. 298	800	10	80
Dialysate medium.			
Sample E. 329	400	25	16
,, E. 346	500	25	20
Undiluted dialysee.			
Sample E. 356	4000	30	130
,, E. 418	2000	20	100
,, E. 438	6000	47	130
,, E. 403	1000	13	120

* The hæmolytic value of a sample of toxin is taken as the reciprocal of the smallest quantity of that toxin, in c.c., which will cause complete hæmolysis of 1 c.c. of a 1 p.c. suspension of washed rabbit's red corpuscles, when heated at 37° C. for one hour.

This indicates that the larger molecules of the medium have some protective action against auto-toxoiding.

TOXOIDING AND PURIFICATION OF "DIALYSATE" TOXOID.

The unfiltered "dialysate toxins" (see Table III) were pooled and formolized (0·3 p.c. of 38–40 p.c. formaldehyde), for six days, by which time the preparation was toxin-free. The antigen-content of the toxoid was determined. To the mass of agar-toxoid 300 c.c. of saline was added, and the mixture was allowed to stand for 24 hours at 0° C. in order to decrease the loss of antigen unavoidably retained in the agar gel. The toxoid was filtered through long cloth. The filtrate was saturated with ammonium sulphate and again filtered through long cloth. The precipitate and cloth were placed in a cellophane bag and dialysed for two days. The volume of the dialysee was noted, and its antigen-content determined as before. The results are recorded in Table IV.

It was found that this toxoid, when saturated with ammonium sulphate,

TABLE IV.—*Toxoiding of "Dialysate" Toxin.*

Material.	Volume.	Antigen value (per c.c.).	Recovery of antigen.
Original toxin	750 c.c.	30 units mean value	..
Raw toxoid	750 ,,	17 units	57 p.c.
Purified toxoid	150 ,,	65 ,,	(*a*) From agar-gel to final product 77 p.c. (*b*) From original toxin to final product 43 p.c.

gave a precipitate grossly in excess of that expected. A fresh quantity of "dialysate medium" was then prepared. This gave no precipitate with ammonium sulphate, but after the agar had been added to the dialysate and then autoclaved, allowed to set, and filtered, a large floccular precipitate was obtained. The precipitate could only be due to either the agar or some impurity in it, and to elucidate the point the following experiments were carried out:

Firstly, the original powdered agar was tested for protein; the result was negative to the following tests—Millon's, xanthroproteic, Hopkins-Cole, biuret and ninhydrin. Then, a 2 p.c. solution of agar in tap-water was prepared, and while still hot, poured into a large dish, so that a sheet of agar 1½–2 mm. deep was obtained. After setting, this was cut up into strips ½ in. by 1½ in., and placed in running tap-water for three weeks, in order to permit any soluble impurities to diffuse out. With boiling distilled water, a 0·3 p.c. solution of the washed agar was made, and allowed to set by cooling to 0° C. for three hours. The soft gel was broken up and filtered. The filtrate was saturated with pure ammonium sulphate, when a floccular precipitate was obtained. This precipitate was found to be soluble in water. The dissolved precipitate was tested for protein by the above tests; the result was negative, but when tested for carbohydrate (Molisch test) a very strong positive reaction was obtained.

These experiments showed that the large floccular precipitate resulting from toxoid prepared on "dialysate medium" by saturation with ammonium sulphate, was very largely material derived from the agar used. The process of preparing an agar-gel, necessarily using heat, appears partially to hydrolyse the agar.

In view of the possibility of the agar precipitate absorbing material from the medium it was considered necessary to ascertain by direct experiment whether all the nitrogenous material of the original medium had been eliminated from the final product. Three g. of agar were added to 500 c.c. of dialysed medium and dissolved by steaming. The medium was autoclaved in 250 c.c. quantities and allowed to set overnight. To one bottle, A, 0·75 c.c. of formalin were added; this was incubated at 37° C. for ten days. To the other bottle, B, 100 c.c. of distilled water were added and the soft agar-gel was broken up by thorough shaking. This was filtered after standing six hours. The filtrate was saturated with ammonium sulphate, when a floccular precipitate formed. The precipitate was collected by centrifugation, and dialysed against running tap-water for two days. The dialysee was labelled D.B. After bottle A had been incubated for ten days, 100 c.c. of distilled water were added and the mixture was then treated exactly like the material of bottle B. The final dialysee was labelled D.A. The volumes of the soft agar filtrates were noted, and the agar-carbohydrate content of the dialysees determined. The results are shown in Table V.

Both dialysees were very pale yellow liquids, containing a slight gelatinous precipitate. Each liquid was tested for protein by the reagents previously mentioned, the result being negative for both; but each gave a strong positive reaction for carbohydrate (Molisch test). Heating an agar-gel for ten days at 37° C., pH 6·5, with 0·3 p.c. formalin, appears to cause hydrolysis, over and

TABLE V.—*Agar-Content of Dialysate Material.*

Material.	Volume of filtrate (c.c.).	Volume of dialysee (c.c.).	Agar-carbohydrate content of dialysee (g.).	Agar-carbohydrate in filtrate (p.c.).
(A) Formolized and incubated	250	47 (D.A.)	0·237	0·095
(B) Not formolized or incubated	200	35 (D.B.)	0·135	0·0675

above that occasioned by steaming and autoclaving. This is concluded from the increase of soluble carbohydrate found in the liquid A.D. compared with B.D.

DIPHTHERIA TOXIN PRODUCTION ON DIALYSATE MEDIUM.

Experiments were carried out to try to produce diphtheria toxin on the "dialysate" medium, with and without added cysteine. The results were unsatisfactory, toxins of very low value being obtained, which were quite unsuitable for practical use.

SUMMARY.

Staphylococcus Wood 46 (and all other toxigenic staphylococci which have been tested) will grow and produce potent toxin on a dialysate medium. The toxin thus produced can be toxoided with formaldehyde.

Such toxoids can be recovered by saturation with ammonium sulphate, by filtering off the precipitate formed, and dialysing it for two days.

Dialysate medium, treated in the same way as toxoid, failed to give a positive reaction for protein.

Toxoid prepared from a medium dialysate and purified by saturation with ammonium sulphate, followed by dialysis of the precipitate, is free from all the nitrogenous constituents of the original medium, and apart from some "water-soluble carbohydrate derived from the agar", the purified toxoid contains only material derived from the result of bacterial activity.

This method of preparing and purifying staphylococcal toxoid (α-antigen) has been repeated many times on a large scale. Toxoids prepared by this means have been found not inferior antigenically to those prepared on the routine medium, and not purified with ammonium sulphate.

The antigenic value, and other properties of purified toxoid, prepared in this manner, such as its relative stability, and its liability to induce reactions when injected into patients, will be the text of a later communication.

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REFERENCES.

BURNET, F. M.—(1932) *J. Path. Bact.*, **35**, 477.
DOLMAN, C. E., AND KITCHING, J. S.—(1935) *Ibid.*, **41**, 137.
GLENNY, A. T., AND STEVENS, M. F.—(1935) *Ibid.*, **40**, 201.
HUGHES, T. P.—(1932) *J. Bact.*, **23**, 437.
KNIGHT, B. C. J. G.—(1935) *Brit. J. exp. Med.*, **16**, 315.

THE TRANSMISSION OF INFLUENZA VIRUS TO HEDGEHOGS.

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THE hedgehog has been shown recently to be susceptible to infection by several filterable viruses. Thus foot-and-mouth disease (Minett, 1927), pseudo-rabies (Remlinger and Bailly, 1933) and yellow fever (Findlay and Clarke, 1934) have all been transmitted to hedgehogs with the production of lesions characteristic of the disease in question.

Preliminary experiments were carried out by the writer in consultation with Mr. J. T. Edwards, M.R.C.V.S., with a view to confirming the experience of the latter (Edwards, 1936) that human acute coryza can be transmitted to hedgehogs. Hedgehogs were inoculated intranasally under ether anæsthesia with unfiltered nasal washings and garglings from individuals suffering from acute common colds. In all, specimens from five patients and from a chimpanzee were tested. Other hedgehogs were inoculated with control material such as broth and washings from a man not suffering from respiratory tract symptoms. In certain instances the hedgehogs were observed to develop a slight nasal discharge and one animal which received a large dose under chloroform anæsthesia became ill. It was not possible, however, to produce constant symptoms or to transmit the condition in series. A possible explanation of the difference between these results and those of Edwards lay in the fact that his animals were infected by *B. bronchisepticus*, while this organism was rarely encountered in the experiments above. A culture of *B. bronchisepticus* alone inoculated intranasally under an anæsthetic gave rise to slight nasal discharge, and there did not appear to be a significant difference between hedgehogs so treated and those receiving human material. Finally histological examination of the turbinates and lungs of the hedgehogs failed to show significant changes.

Next an attempt was made to infect the hedgehog with influenza virus of human origin which had been passed through ferrets and then mice (W.S. strain of Andrewes, Laidlaw and Smith, 1934). It had been found previously by these authors (unpublished observation) that influenza virus would survive in the hedgehog, and this observation was confirmed. Serial passage proved successful, and it was found that the hedgehogs developed a mild disease of the respiratory tract with eventual recovery.

TRANSMISSION BY DIRECT INOCULATION.

The results of the serial passage experiments are shown in Table I. The methods used were as follows :

In each case the animal was inoculated intranasally with 0·25 c.c. of material. Ether anæsthesia was used for the first six hedgehogs, the last four being inoculated without an anæsthetic. The animals were killed with coal gas about the 4th day, and after naked-eye examination portions of the nasal

turbinates and occasionally of the lungs were taken for section. An emulsion was made of the turbinates and sometimes of the lungs by mincing, grinding with powdered pyrex glass, and making up to a 5 p.c. suspension with broth and added Ringer's solution. The supernatant fluids of the emulsions after light centrifuging were inoculated into groups of mice, 0·05 c.c. being introduced intranasally under ether anæsthesia. The mice were killed on the 3rd day after inoculation and the lungs were examined macroscopically for lesions. The first hedgehog was inoculated with mouse passage virus which had undergone 166 passages in ferrets followed by 81 in mice. The virus was obtained as a filtrate from the lungs of mice previously infected with the W.S. virus, filtration being accomplished by means of a gradocol membrane of 0·64μ average pore diameter (Elford, 1931). Subsequent hedgehogs were inoculated with unfiltered turbinate emulsion from the previous hedgehog.

TABLE I.—*Serial Passage of Influenza Virus in Hedgehogs.*

Hedgehog.	Inoculation material.	Anæsthetic.	Symptoms.	Day of killing for passage.	Mouse lesions* from—	
					Turbinates.	Lungs.
No. 1	Mouse lung filtrates	Yes	None	4th	330	222
„ 2	Turbinates from 1	„	„	„	444444	..
„ 3	„ 2	„	„	2nd	442233	..
„ 4	„ 3	„	Slight nasal discharge	4th	034243	..
„ 5	„ 4	„	Ditto	3rd	433233	..
„ 6	„ 5	„	„	4th	4†4†4†4†2†2	222210
„ 7	„ 6	No	None	„	222322	440223
„ 8	„ 7	„	„	„	0222	442233
„ 9	„ 8	„	Slight nasal discharge	6th	4†34†	422
„ 10	„ 9	„	Sneezing. Nasal discharge +	14th—no passage	000000	000000

* The number refers to the size of the lung lesion in each mouse. 1. Lesion 1 mm. in size. 2. Lesion 2–3 mm. in size. 3. Extensive lesions. 4. Complete or almost complete hepatization.
† The lesions in these mice were atypical and greyish—see text.

Table I shows that the mice inoculated with hedgehog material developed lung lesions typical of influenza, with the exception of those receiving material from the 10th hedgehog. This animal was kept till the 14th day after inoculation before killing; in this series and in subsequent experiments virus could be recovered on the 6th and 7th days after inoculation, but not on the 14th and 16th days. Proof that the lung lesions in the mice were due to influenza virus was established by preparing a filtrate from the lungs of mice inoculated with turbinate emulsion from the 6th passage hedgehog. The filtrate produced typical lesions in further mice and was neutralized by hyper-immune anti-influenzal horse-serum (IH_2 of Laidlaw, Smith, Andrewes and Dunkin, 1935).

The first three hedgehogs in the series showed no symptoms, but subsequent animals developed a slight nasal discharge containing polymorphonuclear leucocytes, while the 10th animal began to sneeze on the 6th day, and developed a muco-purulent discharge which dried as a crust on the nostrils. Naked-eye examination of the hedgehogs after killing revealed slight reddening of the nasal turbinates with a little excess of mucus, while the trachea, bronchi and

lungs were normal except in the 9th hedgehog. This animal showed a few hyaline translucent areas near the roots of the lungs. In view of the absence of lung lesions in most of the hedgehogs it was surprising that virus was recovered from the lungs so frequently, but this is also the finding in the case of many ferrets inoculated with influenza virus (Smith, Andrewes and Laidlaw, 1933). The histological changes in the turbinates and lungs will be considered later.

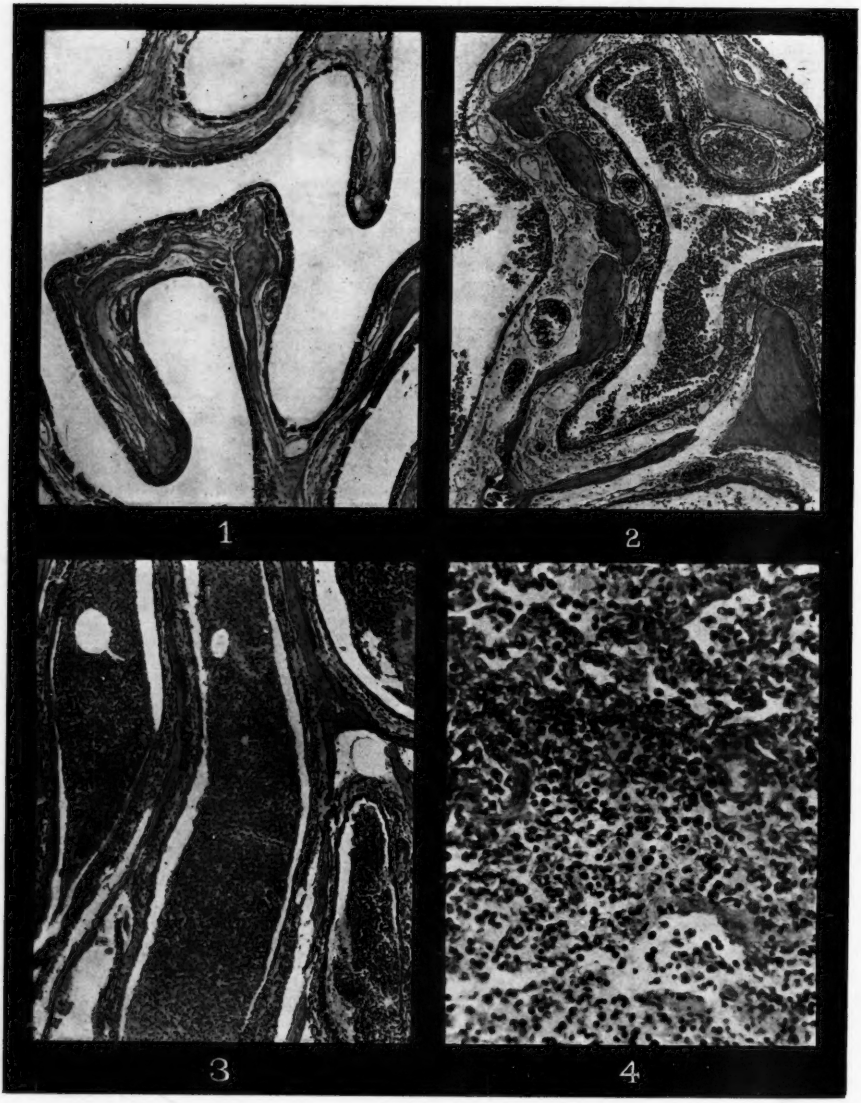
Cultures of the emulsions were made as a routine upon ordinary laboratory media. Those of the lungs gave at most a few colonies of staphylococci, while the turbinates gave a growth of staphylococci and diphtheroid bacilli except in two instances. In hedgehogs 6 and 9, in addition to staphylococci and diphtheroid bacilli, a heavy growth of a Gram-negative bacillus resembling but not typical of *B. bronchisepticus* was obtained. Mice inoculated with turbinate emulsions containing this organism showed atypical greyish lesions, but in each case the lung emulsions from the same hedgehogs produced typical influenzal lesions in mice (Table I). There was therefore some tendency for the turbinate emulsion to become progressively richer in bacteria as passage without filtration continued, but no one organism predominated, while the virus was present in abundance in the lungs where bacteria were scanty.

TRANSMISSION BY CONTACT INFECTION.

In two instances it was shown that a hedgehog could be infected with virus by contact with an infected animal. In the first experiment (Table II) a hedgehog was inoculated with turbinate emulsion from an infected animal and a second hedgehog was placed in the same cage 24 hours later. The directly inoculated animal showed intense sneezing on the 6th day with a muco-purulent nasal discharge, while the other animal showed no symptoms. The animals were killed on the 7th day and virus was recovered from the lungs and turbinates of both. Histologically the lungs and turbinates of the contact animal were normal, though the turbinates of the directly inoculated animal showed lesions characteristic of the infection. The second experiment consisted in placing a hedgehog in the cage of an animal directly inoculated with virus one hour previously, and then keeping both animals for two weeks in order to see whether the contact animal would develop an inapparent infection and would carry the virus for some time. The directly inoculated animal began to sneeze on the 7th day and continued to do so at intervals until the 16th day, when it was killed. The turbinates showed but slight microscopical changes, the lungs were normal, and mice inoculated with

TABLE II.—*Infection of Hedgehogs by Contact.*

	Hedgehog.	Method of inoculation.	Symptoms	Day of killing.	Mouse lesions from—	
					Turbinates.	Lungs.
Experiment 1	No. 11	Emulsion of turbinates. Hedgehog 6 under ether intranasally	Sneezing and nasal discharge	7th	444022	444222
	„ 12	Contact with 11 for 6 days	None	6th	210012	432022
Experiment 2	„ 13	Emulsion of turbinates. Hedgehog 11 under ether intranasally	Sneezing and nasal discharge	16th	000000	000000
	„ 14	Contact with 13 for 16 days	None	„	000000	000000



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material from this animal developed no lesions. The contact animal showed no symptoms and was killed on the 16th day. The turbinates were normal histologically, but the lungs showed brown translucent consolidation of the right middle lobe and root of the azygos lobe. Virus was not recovered from either turbinates or lungs, but examination of the serum of this hedgehog showed that infection by the virus had indeed occurred and had led to the production of antibodies (*vide infra*).

HISTOLOGY OF THE INFECTION.

The nose.—The turbinates of the first few hedgehogs in the series of passage experiments showed congestion and increased numbers of polymorphonuclear leucocytes in the ciliated epithelium. Well-marked changes were present by the fifth passage and consisted of congestion, necrosis of the ciliated epithelium in places, with loss of cilia and even desquamation, and the formation of an exudate of polymorphonuclear leucocytes, red blood-cells and necrotic débris in the lumen. These lesions closely resembled, but were somewhat less extensive than, those found in the turbinates of ferrets infected with influenza virus (Figs. 2, 3). They were present from the 4th to the 7th day after infection of the hedgehog and had disappeared by the 14th day when the epithelium was in an active state of repair. Inclusion bodies were not demonstrated in the nasal mucosa.

The lungs.—The trachea and bronchi were normal in all the hedgehogs and the lungs were also normal except for two instances. One, hedgehog 9 of the passage series, showed on naked-eye examination a few translucent areas, and the other, the contact animal described above, showed more extensive consolidation. Histologically the lesions were the same in the two hedgehogs, consisting of thickening and vacuolation of the alveolar wall, monocytic infiltration, and a scattering of polymorphonuclear and eosinophil leucocytes through the consolidated area and in the lumen of the bronchioles (Fig. 4). Such lesions are found in ferrets infected with virus under an anæsthetic.

SEROLOGICAL CHANGES.

In the ferret, recovery from influenza is accompanied by disappearance of the virus and the development of antibodies in the blood (Smith, Andrewes and Laidlaw, 1933). The demonstration of antibodies in hedgehogs after and not before infection completes the analogy between the diseases in the two animal species. Table III shows the protocols of the experiments, the technique of which was identical with that described by Laidlaw, Smith, Andrewes and Dunkin (1935). The control hedgehog, an animal inoculated with human "cold" material, and a hedgehog bled 6 days after infection had no neutralizing power for the virus. The hedgehogs bled 14 and 16 days

FIG. 1.—Normal hedgehog turbinate. × 53. Weigert's iron-hæmatoxylin and acid fuchsin.

FIG. 2.—Influenza lesion in hedgehog turbinate. × 53. Weigert's iron-hæmatoxylin and acid fuchsin.

FIG. 3.—Influenza lesion in ferret turbinate. × 53. Hæmatoxylin and eosin.

FIG. 4.—Influenza lesion in hedgehog lung. × 220. Hæmatoxylin and eosin.

after inoculation had antibodies equivalent in titre to that of the standard hyperimmune horse-serum, while the animal infected by contact showed antibodies in about one-fifth of the concentration of the horse-serum.

TABLE III.—*Antibodies in Sera as Shown by Mouse Titration.*

Dilution of serum.	1/2.	1/10.	1/50.	1/250.
Immune horse-serum IH_2	..	000	000	000
Control hedgehog	344	334	344	..
Hedgehog 9	334	333	444	..
IH_2 serum	..	000	020	223
Hedgehog 10	000	000	02*0	..
„ 13	000	000	200	..
„ 14	000	002	222	..

* Atypical lesion.

Note.—The numbers show the size of the mouse lung lesions. 2. 2–3 mm. 3. Extensive. 4. Complete hepatization.

SUMMARY.

It has been shown that influenza virus will produce a mild respiratory infection in the hedgehog. The condition is contagious and has been transmitted in series by direct inoculation through 10 hedgehogs. Recovery from the infection is accompanied by the development of antibodies in the blood, while the virus can no longer be recovered from the respiratory tract. There is no evidence that the hedgehog can develop an inapparent infection and can carry the virus for long periods. No opportunity has yet arisen to test the possibility of direct transmission from human beings with influenza to the hedgehog.

Representatives of the primates, the carnivores and the rodents have been known for some time to be susceptible to influenza virus and to these must now be added a representative of the insectivora—the hedgehog. It is of interest that influenza in the hedgehog resembles the disease as seen in the ferret rather than that in the mouse.

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REFERENCES.

ANDREWES, C. H., LAIDLAW, P. P., AND SMITH, W.—(1934) *Lancet*, **2**, 859.—(1935) *Brit. J. exp. Path.*, **16**, 566.

EDWARDS, J. T.—(1936) *Proc. Roy. Soc. Med.*, **29**, 393.

ELFORD, W. J.—(1931) *J. Path. Bact.*, **34**, 505.

FINDLAY, G. M., AND CLARKE, L. M.—(1934) *Trans. R. Soc. trop. Med. Hyg.*, **28**, 193, 335.

LAIDLAW, P. P., SMITH, W., ANDREWES, C. H., AND DUNKIN, G. W.—(1935) *Brit. J. exp. Path.*, **16**, 275.

MINETT, F. C.—(1927) '2nd Progress Report Foot-and-Mouth Disease Committee', London, p. 18.

REMLINGER, P., AND BAILLY, J.—(1933) *C. R. Soc. Biol.*, Paris, **113**, 557.

SMITH, W., ANDREWES, C. H., AND LAIDLAW, P. P.—(1933) *Lancet*, **2**, 66.—(1935) *Brit. J. exp. Path.*, **16**, 291.

ANAËROBIC PNEUMOCOCCUS.*

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In 1897 Rist isolated an anaërobic coccus from a metastatic bone abscess secondary to a bronchiectasis. He referred to the coccus in 1905, and in a personal communication to Prévot (1933) he described the morphological and cultural characters of the organism as corresponding with those of the pneumococcus. Bolognesi in 1907 independently described a similar organism causing an epidemic of pleuropneumonia in laboratory animals, noting also a tendency in his later passage and culture strains to adapt themselves to aërobic cultivation. In neither case did these authors resort to the tests currently employed to identify a pneumococcus. Prévot (1933) has, however, accepted these organisms as varieties of pneumococcus and classifies them as *Diplococcus paleopneumoniæ*. He has not succeeded in isolating strains corresponding with the description he gives of this species. He suggests that the evolution of aërobic bacteria from a more elementary type of anaërobe will account for its disappearance and the failure to isolate it since 1907.

The purpose of this paper is to show that virulent anaërobic pneumococci exist, and to suggest that anaërobic cocci deserve more attention than they have received during recent years. Two of the strains, "ZA" and "ZB", described in this paper, were isolated on separate occasions from the respiratory tract of a man suffering from "chronic bronchitis". A third strain "L" was present in pure culture in the lungs of an individual dying of pneumonia, and on isolation it agreed exactly with strains "ZA" and "ZB"; the antigenic relationships were not studied at that time. Strain "L" has since lost its virulence for mice and efforts to restore virulence have been unsuccessful. The strain has become difficult to dissolve in bile, and, although it is still a strict anaërobe, will not be discussed further in the present paper.

MORPHOLOGY AND STAINING.

The organism is strongly Gram-positive, and ageing of the culture, up to one week, does not produce obvious alteration in the Gram reaction. This is significant in view of the well-known relation between autolysis and reversal of the Gram reaction. In cultures the shape is more or less lanceolate, and the usual arrangement is in pairs. The injection of virulent cultures into mice gives rise to chains of varying length in the pleural and peritoneal fluids (Fig. 1), but in the blood of such mice pairs are the rule. A capsule is easily

* These studies were carried out with the aid of a grant from the Banting Research Foundation Toronto, Canada.

demonstrable in fresh or stained preparations (Fig. 1). Aberrant forms appear in the pleural fluid of mice dying after intraperitoneal injection of strain " ZA " ; one coccus of a pair may be two or three times the size of the other, or there may be incomplete division, giving an irregular rod-shaped organism, but films from the blood and peritoneal cavity of these mice show the normal morphology. It is difficult to account for these morphological peculiarities in terms of the degree of oxygenation of the particular environment, and cultures from such pleural fluid yield only the normal colony types.

Strain " ZB " does not show this feature at all.

CULTURAL CHARACTERS.

Growth does not occur aërobically, except in the case of certain variants to be described later. Cultures were incubated in a McIntosh and Fildes jar.

Surface cultures.—Colonies after 24 hours' incubation on nutrient agar containing 4 p.c. fresh human or rabbit blood vary a little in size according to the dryness of the medium. They are uniform in size on any one plate and about 0·5 mm. diameter as a rule. The surface is smooth, shiny and convex, so that reflections of window bars are sharp. The edge is regular (Fig. 2). The colony is almost transparent, watery in consistency, and easily emulsified in water or saline.

When complete anaërobiosis is obtained within the jar, there is no colour change in the blood, but within a few minutes after exposure to air, a slight amount of green pigmentation develops under and immediately around the colony. On further incubation, autolysis takes place, with increase in diameter of the colony, so that after 48 hours the appearance is as shown in Fig. 3. Occasionally there is a slight degree of hæmolysis around the colonies, and this appears to be related to the susceptibility of the cultures to autolysis.

After 4 or 5 days incubation there is a constant difference in the appearance of the colonies of the two strains. Colonies of strain " ZA " become very flat and papillæ are scattered irregularly over the surface (Fig. 4). Colonies of strain " ZB " retain a peripheral raised margin and papillation is uniform and less marked (Figs. 5 and 6). The edges of the colonies become more and more lobate. Many strains of ordinary pneumococcus, grown anaërobically for similar periods of time, yield colonies closely resembling the foregoing.

Ready growth is obtained anaërobically on the laboratory infusion agar

FIG. 1.—Pleural exudate of mouse, strain " ZA ", Muir's capsule stain. × 1500.

FIG. 2.—Anaërobic surface colony on blood-agar plate, 18 hours' cultivation, strain " ZA " or " ZB ". × 30.

FIG. 3.—Anaërobic surface colony on blood-agar plate, 48 hours' cultivation, strain " ZA " or " ZB ". × 30.

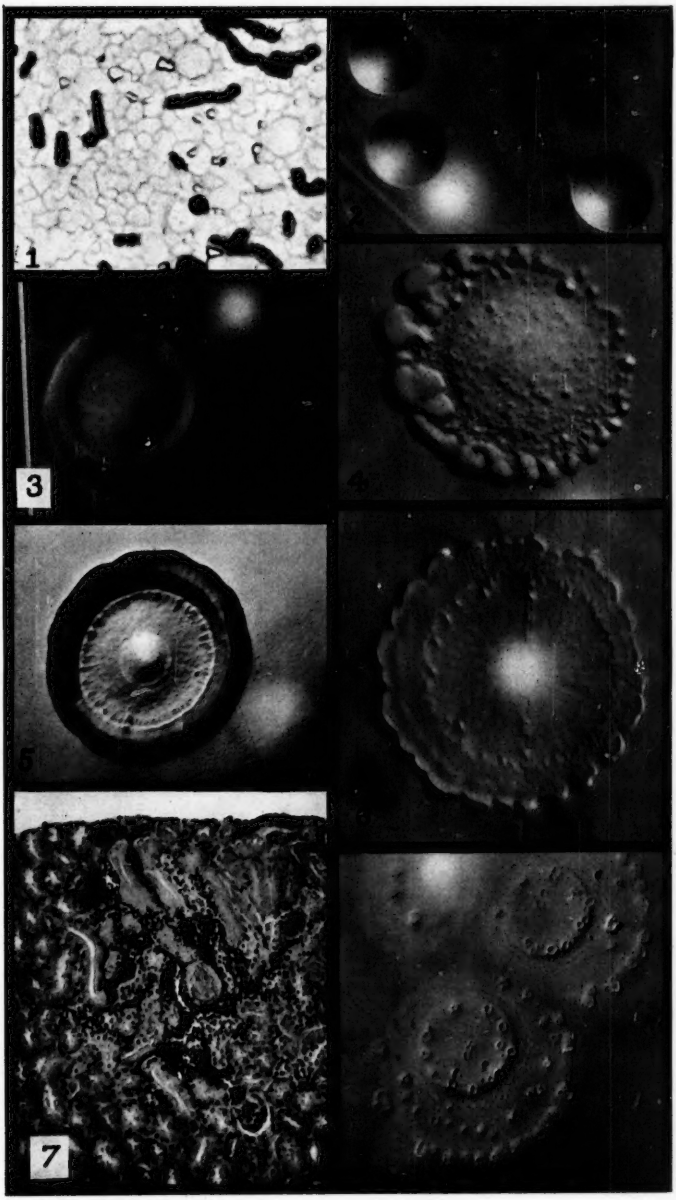
FIG. 4.—Anaërobic colony on blood-agar plate, 5 days' incubation, strain " ZA ". × 30.

FIG. 5.—Anaërobic surface colony on blood-agar plate, 4 days' incubation, strain " ZB ". × 30.

FIG. 6.—Anaërobic surface colony on blood-agar plate, 6 days' incubation, strain " ZB ". × 30.

FIG. 7.—Section of kidney of mouse infected with strain " ZB ". × 100.

FIG. 8.—Strain " ZA " aërobic variant incubated anaërobically 7 days. Blood-agar plate culture. × 30.



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(" E.H." agar of Murray and Ayrton (1924) with 1 p.c. proteose peptone at pH 7·2) and on Dorsett's egg medium, the growth resembling that of the growth of ordinary pneumococcus. Broth cultures yield a turbid growth with some flocculent deposit after 24 hours. The organisms grow well in litmus milk.

Viability.—Cultures remain viable on the surface of blood-agar for at least 6 months under anaërobic conditions at 37° C., even although the growth appears completely autolysed. There is progressive loss of virulence with ageing of the cultures, and aërobic variants make their appearance. The organisms survive drying by the method of Swift (1921) without loss of virulence.

BILE SOLUBILITY.

A saline suspension of growth scraped off the surface of a 24-hour blood agar plate culture lyses completely in 10 minutes at 37° C. in 1 : 1000 sodium desoxycholate. Lysis takes place with equal ease with ox-bile or crystallized bile-salts.

BIOCHEMICAL REACTIONS.

These were carried out in Hiss serum water made from formolized serum (Nicolle, Debains, Jouan, 1918) containing 1 p.c. of fermentable substance and Andradi indicator, sterilized by autoclaving for 20 minutes at 120° C. Dextrose, galactose, maltose, lactose, saccharose, raffinose and inulin were fermented, usually within 24 hours, without the formation of gas and with coagulation of the serum. Acid and clot were formed slowly in salicin; acid only in sorbitol and glycerol; and there was no change in mannitol. In lævulose strain " ZA " produced acid and clot while " ZB " produced acid only.

SEROLOGICAL REACTIONS.

Immune sera were prepared in rabbits by the intravenous injection twice weekly of 18-hour broth culture sterilized by heating at 60° C. for 1 hour. A marked Neufeld reaction is given with homologous culture or mouse peritoneal washings. Agglutination tests were carried out on living broth cultures grown for 18 hours at 37° C. in 5 p.c. rabbit-serum broth pH 7·8. The tests were incubated 4 hours at 37° C., and a final reading was made after the tubes had stood overnight at room temperature. The results are shown in Table I. Agglutination was definitely of the smooth type. It is apparent that strains " ZA " and " ZB " are quite distinct serologically, and except for the relation between " ZB " and type 16, both strains are new serological varieties without relation to other known types.

ANIMAL EXPERIMENTS.

Strains " ZA " and " ZB " are both pathogenic for mice.

" ZA ": This strain is readily raised in virulence by passage through mice, and the cultures used in most of these experiments have killed mice weighing 25 g. within 2 days of receiving 10^{-6} c.c. of an 18-hour serum broth culture intraperitoneally.

TABLE I.

Serum.	Antigen.	Titre of serum.
"ZA"	"ZA"	1/32
..	"ZB"	0
..	Types 1–32*	0
"ZB"	"ZB"	1/128
..	"ZA"	0
..	Type 16	1/32
..	Remaining types	0
"ZB" absorbed with type 16	"ZB"	1/128
..	Type 16	0

* The pneumococcus types 1–32 were obtained through the courtesy of Dr. Georgia Cooper.

At autopsy the pleural and peritoneal cavities contain pale straw-coloured serous fluid, with a tendency to clot, in amounts much greater than the pneumococcus ordinarily produces. The fluid contains abundant organisms and fair numbers of both polynuclear and mononuclear cells. The morphology of the cocci in the pleural fluid is atypical (*vide supra*). The spleen is enlarged and slightly darker in colour than normal, but there is no other marked pathological change in the organs. Organisms can be seen in smears and cultivated from the heart's blood, and with strains of this degree of virulence aërobic cultures on blood-agar plates or in broth usually remain sterile. Rarely an occasional colony will grow aërobically on several days' incubation.

"ZB": Repeated mouse passage has not raised the virulence of this strain for mice beyond a minimal lethal dose of 10^{-3} c.c. of an 18-hour broth culture, and even this degree of virulence is readily lost in culture.

At autopsy the mice injected with "ZB" present differences from those injected with "ZA" or with ordinary pneumococcus culture. No morphological peculiarities in the cocci are observable, but despite the intraperitoneal route, washings of the peritoneal cavity are practically devoid of cocci, even though the pleural fluid and blood contain enormous numbers. No explanation has been found for the clearing of the peritoneal cavity, and the omentum does not appear to be involved in the process. The spleen is enlarged and slightly darker in colour than the normal. The kidneys of mice killed by "ZB" infection are pale, with a few punctate hæmorrhages. Histologically the kidneys show focal lesions in many of the glomeruli; the cells of the first convoluted tubules are degenerate; the nuclei do not stain, the cytoplasm is hyaline and the lumen of the tubule is obliterated. The whole lesion is heavily infiltrated with leucocytes (Fig. 7). In sections stained by Glynn's method (1935), Gram-positive diplococci can be seen, filling the capillaries of the glomeruli and the space within Bowman's capsule, and also in the damaged tubules. Organisms can be found only on careful search in the other organs. No obvious macroscopic lesions were seen in other organs and tissues.

Neither strain is pathogenic for the guinea-pig in doses up to 2 c.c. of broth culture, *i. e.* 2,000,000 mouse infective doses of "ZA" and 2000 of "ZB". Three rabbits were injected with 0·1 c.c. of an 18-hour broth culture of strain "ZA", intravenously, subcutaneously and intracutaneously respectively. All the animals survived. The rabbit receiving the intracutaneous injection showed for 2 days an indurated, erythematous lesion which healed completely. Three rabbits similarly inoculated survived 1 c.c. of an 18-hour broth culture of strain "ZB". The resistance of the guinea-pig to experimental pneumococcus infection is notorious, but the disproportion between the lethal doses for mice and rabbits in these two strains is unusual.

VARIATION.

Old stock cultures sealed in anaërobic jars and kept at 37° C. give rise to aërobic variants with progressive loss of virulence. Repeated mouse passage with minimal infective doses of these stock cultures eliminates the variants and restores virulence. These aërobic variants retain evidence of their source in so far as they grow much better anaërobically than aërobically. At least two different aërobic strains have been derived from "ZA" by colony selection.

Variant (1).—The colony autolyses very slowly. The organisms have the typical morphology and are capsulated. The culture agglutinates to titre ("S" type) with the homologous serum and gives also the Neufeld "Quellung" reaction. The strain is slightly virulent for mice, but mouse passage does not restore strict anaërobic properties. Elimination of this variant by mouse passage from stock cultures containing it probably depends on the smaller degree of virulence and the small numbers of the variant.

Variant (2).—The colony autolyses readily. Morphology is typical, capsules are not demonstrable, and agglutination is of the "R" type. Mice survive 1·0 c.c. of an 18-hour broth culture intraperitoneally.

These variants are bile-soluble, and their biochemical reactions are identical with those of the parent culture. When these two strains are cultivated anaërobically the colonies become indistinguishable from each other, and resemble closely the colonies of the virulent, strictly anaërobic, parent culture. The relation of these variants to the rough variant of the pneumococcus is being examined further.

The attempt has been made to restore the anaërobic requirements in these variants. Grown for 24 hours aërobically and then placed in a McIntosh and Fildes jar under anaërobic conditions, the colony after 1 week appears quite flat with numerous papillæ on the surface and at the periphery (Fig. 8). Subcultures from these papillæ will grow only anaërobically. After the first anaërobic subcultures, however, anaërobic requirements are lost again, and it has so far been impossible to reverse the respiratory variation beyond the first subculture.

DISCUSSION.

The morphology, solubility in bile, biochemical characters, and pathogenicity for mice are characteristic of the pneumococcus. Serologically these strains are new varieties, and differ further from the classical pneumococcus in their respiratory requirements. Whether or not we accept the current explanations of anaërobic respiration in terms of peroxide and catalase, the dissociation of these organisms to aërobic types and back again involves considerations of loss and gain of respiratory properties which merit further study. Moreover, if, as appears probable, the anaërobic character is associated with the somatic portion of the cell, there would seem to be no reason why a series of anaërobic pneumococci, analogous to the aërobic series, should not be created in the laboratory or arise in nature.

Observers have disagreed as to whether or not the daughter colonies arising in old autolysed cultures are variants. The disagreements appear to depend rather on the definition of the term "variation". Atkin (1926)

demonstrated the insolubility in bile of the cells comprising the daughter colony, and although such cells give normal subcultures, he considered them to be variants. Griffith (1928) believed that the papillæ arising on smooth colonies were composed of rough organisms, but Klumpen (1932) showed that such daughter colonies gave rise to smooth virulent subcultures. The experiments described in this paper support both points of view. The papillæ on anaërobic culture do not differ from the parent culture, but the papillæ derived by subsequent anaërobic cultivation of the aërobic variants consist, at least for the first subcultures, of strictly anaërobic organisms. This at any rate is a temporary physiological variation.

The pathological changes in the kidneys of mice infected with strain " ZB " are not seen in infections of these animals with the ordinary pneumococcus. The lesions are always in strict relation to focal deposits of micro-organisms, and appear to originate in the glomeruli. It seems most probable that the focal character of the lesions is due to local action of the organisms rather than to a concentration of toxic materials by excretion and absorption.

SUMMARY.

1. Two strains of anaërobic pneumococci are described.
2. The strains serologically are new varieties.
3. One of the strains produces focal lesions in the kidneys of mice.
4. The strains give rise to aërobic variants, and the variation is reversible.

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REFERENCES.

Atkin, E. E.—(1926) *Brit. J. exp. Path.*, **7**, 167.
Bolognesi, G.—(1907) *Zbl. Bakt.*, Abt. I, Orig. **43**, 113.
Glynn, J. H.—(1935) *Arch. Path.*, **20**, 896.
Griffith, F.—(1928) *J. Hyg.*, Camb., **27**, 113.
Klumpen, W.—(1932) *Zbl. Bakt.*, Abt. I, Orig., **124**, 241.
Murray, E. G. D., and Ayrton, R.—(1924) *J. Hyg.*, Camb., **23**, 23.
Nicolle, M., Debains, E., and Jouan, C.—(1918) *Ann. Inst. Pasteur*, **32**, 150.
Prévot, A. R.—(1933) *Ann. Sci. nat.* (*Botanique*), **15**, 23.
Rist, E.—(1905) *Bull. Inst. Pasteur*, **3**, 50.
Swift, H. F.—(1921) *J. exp. Med.*, **33**, 69.